STUDY OF STRESS AND OTHER UNCONTROLLED FACTORS IN ANIMAL EXPERIMENTATION

by

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RESUME

In rodents, traditional animal handling and conventional animal housing produce psychosocial and anxiety stress with the induction of easily measurable physiological changes. One of the more serious is a reduction of immunocompetence. Thus, any environmental circumstance which generates either acute or chronic stress in animals may compromise the data of otherwise carefully planned and executed experiments. The findings of most relevance to biomedical research requiring the use of rodents, are the data resulting from the re-examination of common laboratory procedures and animal housing, which cause important alterations in physiological and immunological parameters. These include the various effects of cage population densities, proximity of males and females to each other, and the consequences of the use of wire-bottom cages. The overall data indicate the need for a systematic re-evaluation of conventional animal housing and experimental techniques commonly employed in biomedical research.

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R: REDACTED MATERIAL

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BUDGET

Project Personnel

Professional Staff

Percent Time		Salary	F/B
50%			
50%			
25%			
25%			
	Sub-Total	46,500	7,300
	Laboratory Personnel		
100%	Laboratory Assistant	10,000	1.500
100%	Biologist (Animal Studies)	10,000	1,500
100%	Biochemical Assistant	10,000	1,500
100%	Primate Biologist	10,000	1,500
50%	Animal Care Technician	4,000	650
50%	Statistician/Draftsman	5,000	750
25%	Secretary/Admin. Assistant)	2,500	400
	Sub-Total	51,500	7,800
	Total	98,000	15,100

$98,000	Total Salaries
15,100	F/B
$113,100	Total Salaries + F/B
10,900	Supplies, etc. (See attachment)
$124,000	Total
31,000	PNRF Overhead at 25% of Budget
$155,000	TOTAL PROJECT BUDGET

[redacted]

The budgets for successive years could be the same as this one, with appropriate cost-of-living increments.

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Budget

Supplies, Instrumentation, Travel, Miscellany

Animals, Food and Bedding	$5,000
Miscellaneous Supplies	1,200
Instrument Maintenance	1,800
Other Costs: Xerox, Telephone, Publication, Laundry, etc.	900
Travel (2 persons, 2 meetings)	2,000
Total	$10,900

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INTRODUCTION AND BACKGROUND

Prior to the initiation of these studies, it was observed in our laboratory, and by others, that a number of uncontrolled environmental circumstances and technical procedures produced physiological alterations in experimental and control animals, and that these uncontrolled stress-associated changes interferred with, or distorted, various experimental parameters that cancer researchers, immunologists, and other investigators were attempting to measure with accuracy. As a consequence, these findings provided disquieting evidence that the most carefully done studies using animal models were subject to misinterpretation, or could yield erroneous data unless such stress-inducing environmental and animal-handling factors could be identified and controlled.

To partially remedy the experimental problems, certain modified animal handling procedures and animal housing have been established in our laboratories, which are now considered essential prerequisites for animal experimentation. These improved techniques allow the investigator to distinguish between truly quiescent and chronically stressed mice, as well as to determine the authentic consequences of experimentally imposed stress (26, 28, 29, 39, 43).

Another experimental factor that is essential to the control and detection of inadvertent stress is the elimination of the generally unrecognized LDH-virus contamination from experimental mice, tumors, and oncogenic virus preparations (30-32, 36). In mice infected with this ubiquitously distributed murine virus, changes occur in plasma corticosterone levels, the thymus, spleen, lymph nodes, macrophages, T cells and B cells, as well as functional components of various enzyme systems,

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These improved experimental conditions and procedures permit the use of more reliable experimental designs for the detection and quantitation of mild stress by facilitating accurate and sensitive measurements of a variety of relevant parameters(31a,34,39-41). These include plasma corticosterone elevation (CSR), T cell and B cell depletion, adrenal hypertrophy, and weight loss of the thymus, spleen, and nodes. It has been confirmed, under more reliable experimental conditions, that the previously observed stress-mediated physiological changes in mice, such as thymus involution, lymphocytopenia, etc., quickly follow and are undoubtedly caused by the rapid and profound elevations of plasma corticosterone (7,25,29,31,34,36).

One of the more important products of these basic protective techniques has been an experimental documentation of the rapid and exquisitely sentitive physiological response of mice to the mild stress that is associated with conventional housing and animal handling procedures employed in most laboratories (25-26,29,39,41,43).

In order to carefully control the intensity of experimentally induced stressful stimuli, a simple device has been designed and tested for inducing mild, non-traumatic anxiety-stress (31a). The manifestations of such stress are quantitative, predictable, and readily reproducible.

As a result of these technique developments, the specific hormonal and cellular changes which occur as a consequence of stress can now be studied with confidence. Of special relevance is the consequential ability to obtain reproducible alterations of some neoplastic processes by either emotionally induced or chemically simulated stress.

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The Biochemical Nature of Stress.

Anxiety, or other varieties of psychosocial stresses in experimental animals, produce a series of biochemical events that are mediated through the neuroendocrine system. These biochemical responses have demonstrable destructive effects upon specific cells and tissues that are required for immunological defense. As a consequence, the stress-compromised animal is less capable of defending itself against cancer cells, infectious agents, and certain other disease processes. Thus, uncontrolled stress factors are important components to be reckoned with in designing and carrying out experiments.

There are, of course, many varieties of stress which may activate separate physiological systems, either singly or as complexes. For purposes of simplification in this report we shall restrict our discussion and data presentation to the effects of uncomplicated anxiety involving emotionally aroused stress which is associated with the activation of the adrenal cortex.

A characteristic expression of such stress is an abrupt and dramatic increase in circulating plasma glucocorticoids. In rodents, this is corticosterone; in man it is cortisol. Such stress-induced hormone elevations produce secondary stress effects involving T cells and thymic components, and thus elements of the immunological apparatus (15,31a,44). Relevant metabolic and related biochemical alterations also occur through the influences of increased glucocorticoid action (47,51).

Within the framework of this limited definition of stress, it is assumed that the adrenal medulla is either not significantly activated by mild stress, or does not greatly alter the effects of the adrenal cortex. However, activation of the adrenal medulla system occurs in rodents under more drastic stressful conditions where fear or rage is the inciter (2,16).

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The rationale of stress-induced or stress-mediated disease follows logically from a series of known physiological events. Although the overall biochemical phenomena associated with stress are complex and have many subtle consequences, the primary events relevant to disease processes appear to be straightforward, at least those involving the adrenal cortex, as depicted in the abbreviated pathway diagram of Figure 1.

Critical experimental studies cannot be carried out with confidence using conventional animal housing facilities, since they are not suitable for the maintenance of normal quiescent baseline values of stress-associated hormonal and cellular elements that influence or control physiological and pathological purposes. We have therefore designed animal facilities which not only serve the experimental needs of our physiological stress research, but in addition, provide safer facilities for working with infectious or allergenic agents. Prototypes of low-stress animal facilities are shown in Figure 2.

From the standpoint of the low stress that is essential for carefully biological research, these individually ventilated shelf units offer several beneficial features (26, 28-31). For example, the enclosed shelves provide a substantial amount of sound-proofing, which is of special significance since it has been established that animals are stressed by a wide variety of noises which stimulate immunologically adverse neuroendocrine reactions. Stressful noises and cage motion are prevalent in most conventional animal rooms, particularly where there are rolling metal racks, metal cages, radios, shouting, frequent cage cleaning with rough handling, and other noisy and stressful operations.

The most essential features required for protective low-stress animal housing are as follows: (a) No recirculation of noxious air that has been in

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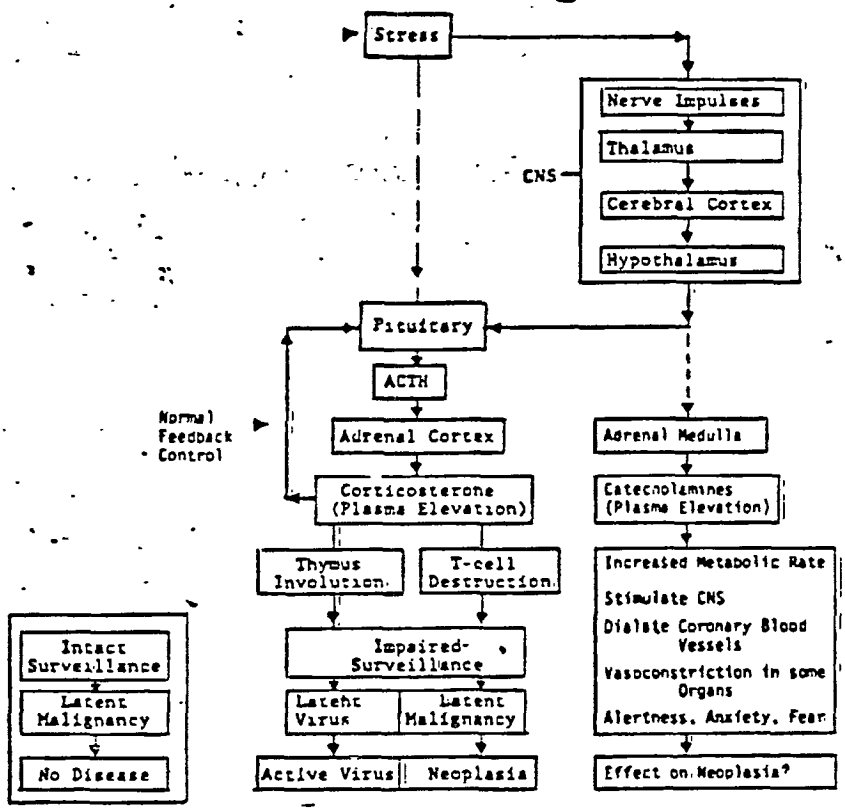

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Figure 1. Abridged diagram of the physiological pathways whereby anxiety stress signals are transmitted to the adrenal cortex, which responds to produce an increased level of adrenal cortical hormone in the blood.

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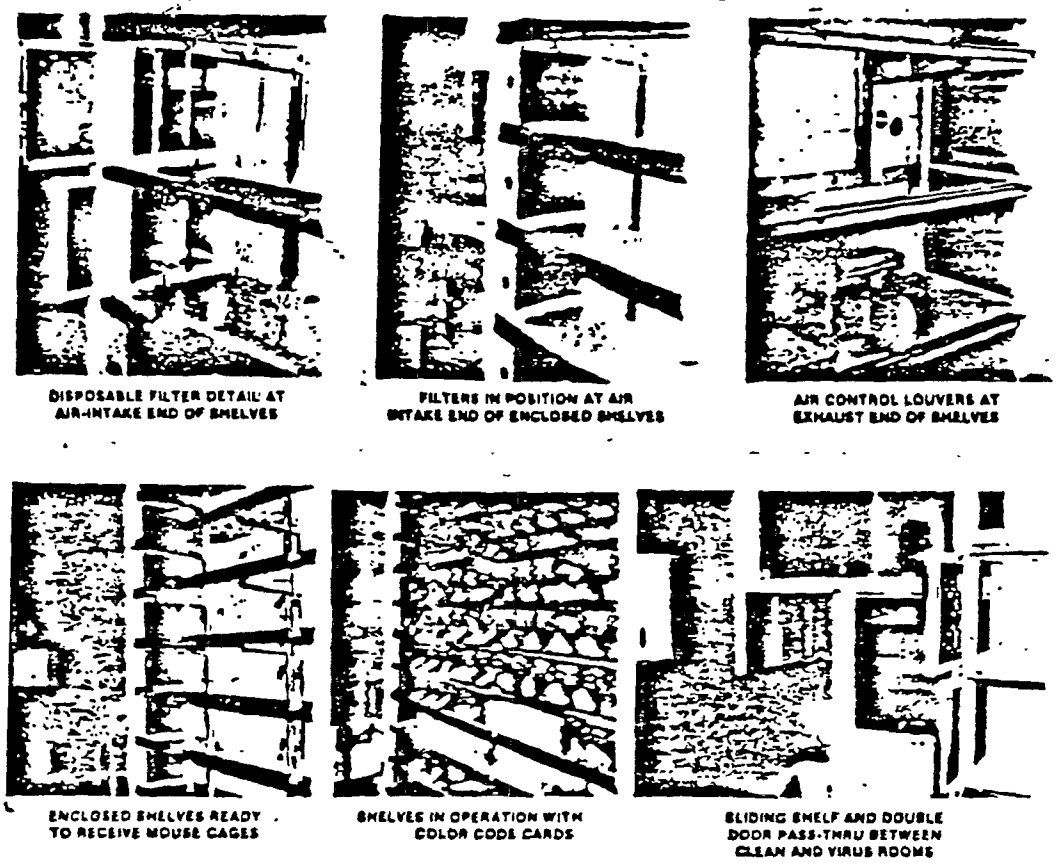
DISPOSABLE FILTER DETAIL AT AIR-INTAKE END OF SHELVES
FILTERS IN POSITION AT AIR INTAKE END OF ENCLOSED SHELVES
AIR CONTROL LOUVERS AT EXHAUST END OF SHELVES
ENCLOSED SHELVES READY TO RECEIVE MOUSE CAGES
SHELVES IN OPERATION WITH COLOR CODE CARDS
SLIDING SHELF AND DOUBLE DOOR PASS-THRU BETWEEN CLEAN AND VIRUS ROOMS

Figure 2. Protective animal facilities. Standard plastic cages are housed within enclosed, ventilated, shelves. Hinged plexiglas doors seal the enclosed shelves, which permit filtered air to be drawn over the cages with a minimum of turbulence. Contaminated air is constantly exhausted by negative pressure and is vented outside of the animal area. These units protect the animals from uncontrolled stress-inducing noises, drafts, thermal and humidity fluctuations, odors, pheromones, and personnel activities. Note that the plexiglas doors are hinged at the bottom. This permits the doors to fold down and to hang vertically without hazard or obstruction when animal cages are being removed or replaced. Negative air pressure and spring action of the hinges seals the doors when in the closed position. These units are built in four-foot sections, and are free-standing for end to end or back to back installation.

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previous contact with the animals; (b) partial sound-proofing of storage shelves to protect the animals from stressful noises; (c) elimination of vibrations and high pitched sounds of centrifuges, vacuum cleaners, and heavy equipment; (d) elimination of drafts and air turbulence; (e) light control to stabilize circadian rhythms; (f) segregation of males and females with respect to transmissible odors, pheromones, and other signals; (d) segregation of experimental animals that are experiencing stress from normal or experimental control animals; (h) introduction of special minimal-stress animal handling techniques and cage cleaning procedures; and (i) avoidance of drafty, uncomfortable and stressful wire-bottom cages.

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ESSENTIAL TECHNIQUES FOR ANIMAL STUDIES

Animal Caging and Environment. Standard plastic cages, 28 x 18 x 13 cm (11 x 7 x 5 inches), containing about 2 cm of San-I-Cel ground corncob bedding, were employed in all experiments unless otherwise indicated. In our experience, plastic cages are superior to metal cages in terms of general insulation, resistance to temperature changes, cold conductivity, and minimization of sound as well as thermal stress factors. Inasmuch as endocrine and various circadian effects are related to light and dark exposures, the plastic cages are also preferable over metal for controlling admittance of diffuse light to the animals. Standard 12-hour intervals of light and dark were controlled by automatic clock-controlled switches. Temperature was maintained at 75°F (± 3).

Housing. All animals, including both stock and experimental groups, except those in special environmental experiments, were protected by a special barrier system consisting of ventilated enclosed shelves provided with filtered laminar air-flow vented outside of the building following contact with the mice and their released aerosols (26). When the doors of these facilities are closed, each shelf becomes a separate, independent and isolated unit within a larger protected facility. The only cross-contamination that is theoretically possible would occur in those cages that are "down-wind" from infected animals in the airflow of a given shelf. However, by appropriate segregation, this possibility is readily avoided.

Mouse Diet and Sanitation. The mouse diet consisted of Wayne Lab Blox pellets supplied through the cage-top food hopper unless otherwise indicated. This nutritionally complete diet was available to the mice ad libitum. Clean water was available through the use of pint glass bottles and stainless steel drinking tubes. High standards of sanitation were employed in the routine changing and sterilization of drinking bottles, tubes, and cages. Cages and bedding were changed once a week with a minimum of animal disturbance.

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Mice. Two C3H mouse substrains were employed in these studies: C3H/Bi, harboring from birth the milk-borne mammary tumor virus (MTV), and C3H/He, derived from cesarean, foster-nursed animals and thus free of high titers of the milk-passaged virus. In certain experiments, BDF hybrid mice (C57BL/6 x DBA/2) were used. All mice were obtained from commercial breeders as weanlings weighing 18 to 22 grams. Upon arrival in our laboratory they were randomly segregated into groups of ten, and housed in standard plastic holding cages. These cage groupings, or further subdivisions into smaller, or recombination into larger groups, were maintained throughout the experimental periods. To minimize psychosocial stress, remixing of mice following their social equilibration was avoided. Experiments were usually initiated when the animals were 8 to 12 weeks of age, weighing 20 to 30 grams, and were fully quiescent and equilibrated in terms of acquired transportation-induced stress.

Experimental Groups. Mice used in a given experiment were from uniform groups in terms of age, weight, sex, strain, and origin. Mice were received from reliable commercial breeders in batches of 50 or 100, with their age range limited to about five days. Upon receipt, the animals were held in protective facilities for a minimum of two weeks equilibration prior to use, usually longer. Any abnormal-appearing mice, or those departing from our weight and other specifications, were discarded. The establishment of mouse social order equilibration is of importance in maintaining animals under conditions of minimum stress in order to avoid elevated adrenocortical concentrations with their attending effects on the thymus, T cells, and immunological competence.

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Tumor Implantation and Measurements. The Gardner lymphosarcoma (6C3HED) grows as a solid tumor mass when implanted subcutaneously (s.c.). The donor tumors were harvested aseptically and injected into recipient mice as a 20 to 50 percent suspension of free cells and small fragments of tumor tissue in 0.9 percent NaCl solution (29). In order to follow the tumor growth and regression quantitatively, the tumor mass was measured in three dimensions by calipers (29). The 6C3HED lymphosarcoma has been maintained in our laboratory for about eight years by intermittent passage in C3H female mice.

Elimination of a Common Tumor Contaminent. Without special processing, most transplantable tumors carry the LDH-virus as a perpetual contamination (30-32). Since the hazards that this virus may cause in the interpretation of data have been distussed and in some cases demonstrated (30-32), all tumors utilized in these studies were processed to remove the LDH-virus. For certain experimental purposes involving biological stress, the virus was intentionally reintroduced into specific mouse groups as appropriate. Such infected mice and non-infected controls were housed and handled so as to avoid subsequent cross-contamination. Critical monitoring of our mice for absence of the virus in the controls was a standard procedure.

Corticoid Injections. Fluocinolone acetonide (FCA), a synthetic corticoid, was administered intraperitoneally (i.p.) as a freshly-prepared saline suspension. Because of limited solubility, the FCA is absorbed slowly to give a long-lasting effect from a single dose of 50 mg/kg.

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Assay for Plasma Corticosterone in Mice. A micro-fluorimetric assay was developed in our laboratories (39), based on the procedure described by Glick, et al. (14). Our assay requires only 50 µl of plasma obtained from individual mice by the non-harmful orbital bleeding procedure (27); repetitive assays were carried out on plasma samples pooled from several mice, depending upon individual experimental requirements.

Amino Acid Determinations. Analysis of free plasma and urine amino acids were carried out on a Beckman Model 120 B amino acid analyzer modified to provide accelerated, semiautomatic runs of high sensitivity (42). Recently revised and improved methods of analysis were employed (38). An improved sodium citrate buffer system for basic amino acids allows the routine analysis of E-N-monomethyllysine along with all other basic amino acids. Lithium citrate buffers were used for the acidic and neutral amino acids.

Organ Weights. The thymus weight responses of mice exposed to environmental or experimental stress were compared with those of appropriate control animals. The control mice were sham-injected with saline in those experiments where treatment injections were given. At autopsy, the weights of the thymus, spleen, adrenals, and pooled inguinal and axillary lymph nodes were determined for each animal. Similar measurements were made following administration of synthetic corticoids, or of any other stress-inducing experimental procedure such as rotational-induced anxiety (29).

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RECENT EXPERIMENTAL OBSERVATIONS MADE IN OUR LABORATORY

Corticosterone Elevation by Stress. Figure 3 illustrates several relevant facts. First that an extremely mild "handling" stress is capable of inducing anxiety in mice which, in turn, produces an immediate and substantial increase in plasma corticosterone. The cluster of unconnected points at the bottom left hand corner of the chart are "normal", quiescent plasma corticosterone values of individual mice, obtained when a group of ten animals were removed from protective storage and all ten bled by the rapid orbital bleeding technique within three and one-half minutes. This short time period is insufficient for the overt expression of any stress or anxiety to be manifested biochemically by an elevation of plasma corticosterone. However, when analogous cages of mice were similarly removed from protective storage, and one animal was captured and bled every two or three minutes, so that the total elapsed handling time was about thirty minutes between the first and last animal captures, the ascending curve shows the rate at which corticosterone becomes elevated in the blood plasma of such mildly stressed animals. Similar results have been obtained from each of four different strains tested (BDF, BAF, C3H, and CBA).

These experimental results further demonstrate the need for quiescent, protective housing facilities, and the necessity to employ special animal handling techniques in studies on biochemical and biological effects of stress.

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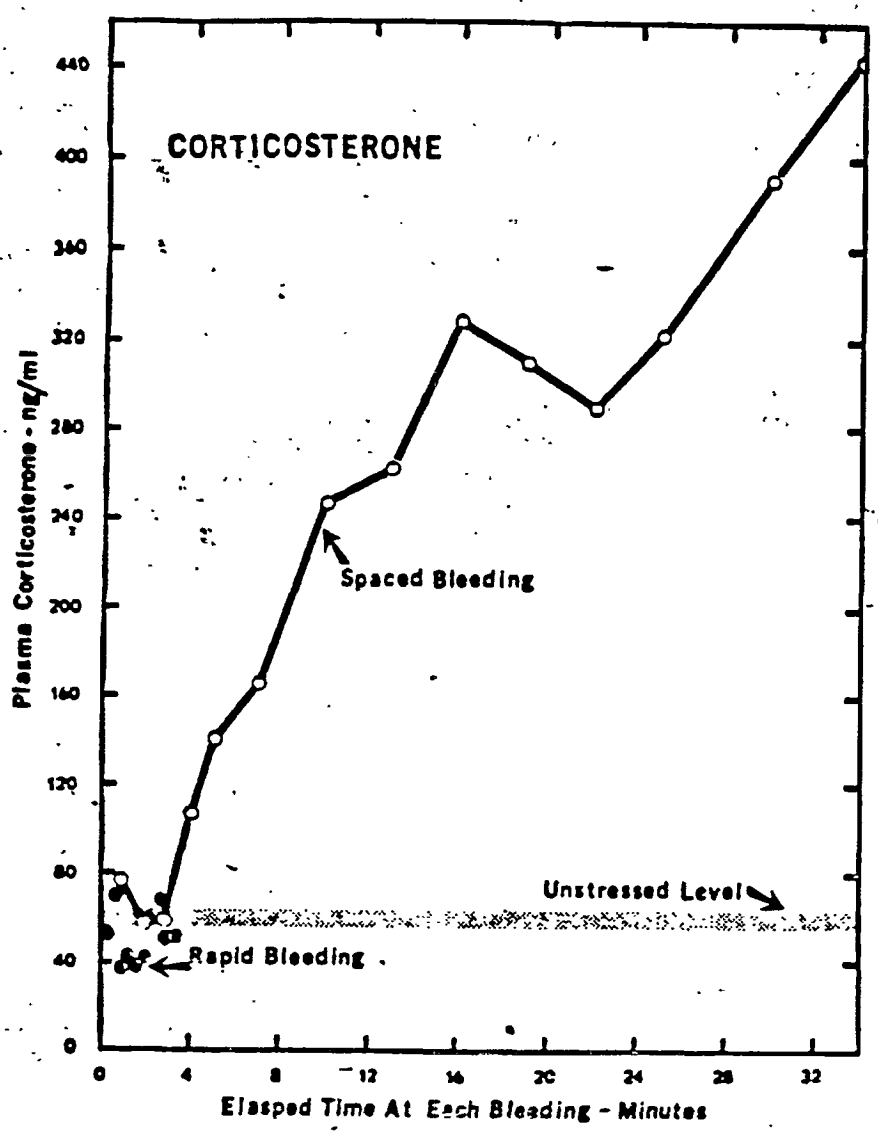

Figure 3. Influence of normal laboratory manipulation upon the production of stress-induced corticosterone levels in normal mice. The cluster of unconnected points at the unstressed level of corticosterone represents ten animals whose blood samples were taken rapidly within a three and one-half minute period. The ascending curve shows the systematic increase in corticosterone with time following removal of the mice from their storage shelf, followed by capturing individual animals in the cage and bleeding at periodic intervals as indicated in the Figure. This demonstrates the rapidity of physiological response to stress as measured by the corticosterone parameter.

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Benign Stress Machine.

Previous studies have demonstrated the need for a simple, non-traumatic and reproducible means for inducing controlled stress in experimental animals. Such stress should be produced without activating other hormonal systems, significantly altering the metabolism of the host, or changing caloric intake. Figure 4 shows a simple stress-inducing device that provides a controlled quantitative form of stress which is readily reproducible, and lends itself to automation with a wide variety of intermittent stress/rest programming. This machine is a modified phonograph turntable that has the four standard speeds of 16, 33, 45, and 78 rpm. The instrument has been designed so that an entire cage of animals can be placed on the machine for rotation without changing the established arrangement of their living facilities. With this device it is not necessary to alter the availability of their food and water, inasmuch as the slow rotational speeds and intermittent on/off cycles permit the animals to move about their cage and to continue eating and drinking. It may also be noted that the lateral gravitational force involved is less that one G. This stress-inducing instrument is thus not a centrifuge device, but merely a mechanical means for inducing mild spatial disorientation, and possibly virtigo or dizziness, with its associated anxiety.

Stress-Induced Corticosterone Quantitation.

The ability of this instrument to induce variable intensities of stress is shown in Figure 5 , where plasma corticosterone values are plotted against the various rotational speeds. It may be seen that following the rotation of separate groups of mice for 20 minutes at each of the four speeds, that a systematic increase in plasma corticosterone was observed (31a,39). For most stress-

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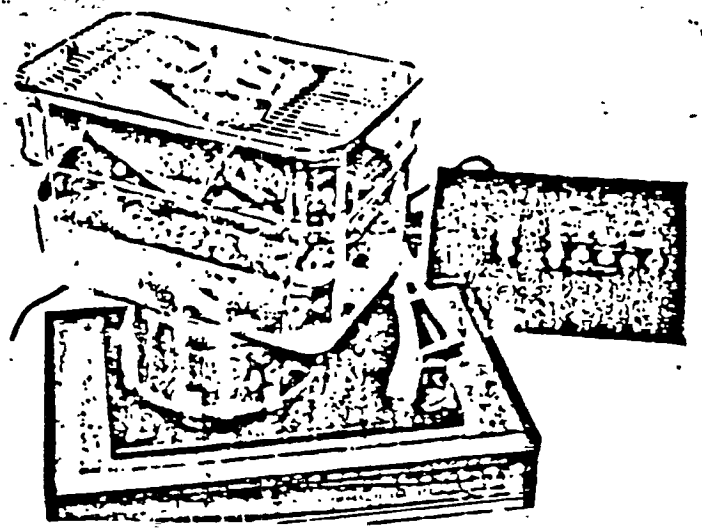

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Figure 4 . A new programmable stress machine. Entire cages of mice are slowly rotated at predetermined speeds and for various periods of intermittent rotation and rest. Automatic timers control the rotation/rest ratios, which permits the mice to be stressed in their cages without depriving them of food or water, or employing physical restraint. The machine can be programmed for any time and periodicity that the experimental design requires.

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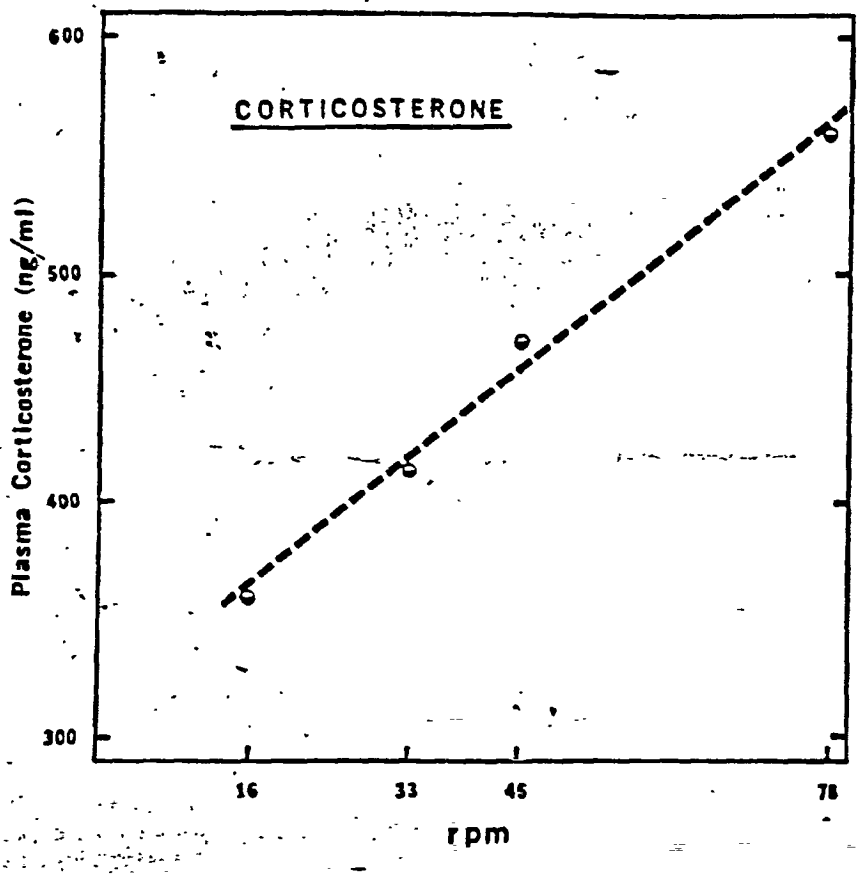

Figure 5 . The relationship between speed of rotation (rpm) and the resulting corticosterone levels in the mouse plasma following 20 minutes of cage rotation. It should be noted that the maximum speed employed generates less than one G at the maximum radius at the cage corners. Thus, this is not a centrifugal-induced stress, but merely a spatial disorientation, which produces anxiety with a consequential corticosterone increase, probably via the hypothalamus, pituitary, and ACTH pathways, which activate the adrenal cortex.

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inducing purposes, the intermediate speed of 45 rpm has been employed, and the animals are usually rotated for 10 minutes followed by a 50-minute rest. This, or other cycles, can be repeated for any desired period of time by employing programmed automation.

Rotational Stress and Cellular Changes.

Figure 6 shows the effect of such programmed rotation on the WBC count. It is relevant to note that this stress-induced leucocytopenia immediately follows, and is a consequence of the elevated plasma corticosterone levels induced by the anxiety stress.

Enhancement of Tumor Growth by Rotational Stress.

Figure 7 shows the influence of intermittent rotational stress on the subsequent growth rate of the Gardner lymphosarcoma (6C3HED) in C3H/He mice. In this experiment the tumor-bearing mice were exposed to intermittent rotational stress (45 rpm) for ten minutes out of each hour during the first six days following subcutaneous implantation of the transplantable lymphosarcoma. A logical interpretation of these observations suggests that those animals receiving rotational stress had some elements of their cell-mediated immunological competence compromised (3,8,17,37), which permitted this sensitive tumor to grow at a more rapid rate than occurred in the control animals, which possessed the immunological capability to restrain the optimal growth of this tumor. Such a stress-induced decrease in immunological competence is a natural consequence of the corticosterone elevation induced by anxiety stress.

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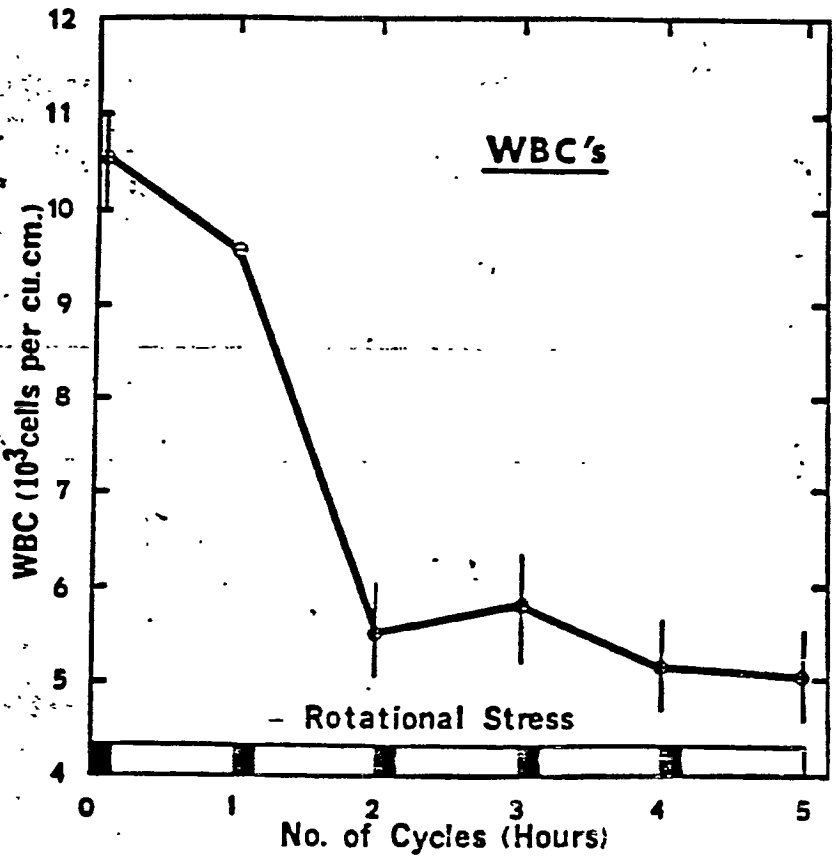

Figure 6, Lymphocytopenia induced by mild, non-traumatic rotational stress at 45 rpm during five 60 minute cycles consisting of ten minutes rotation followed by 50 minutes rest. An approximate 50 percent lymphocytopenia is produced by the end of the second cycle, and is maintained throughout the five hours of intermittent stress. Standard errors for each point are represented by vertical lines.

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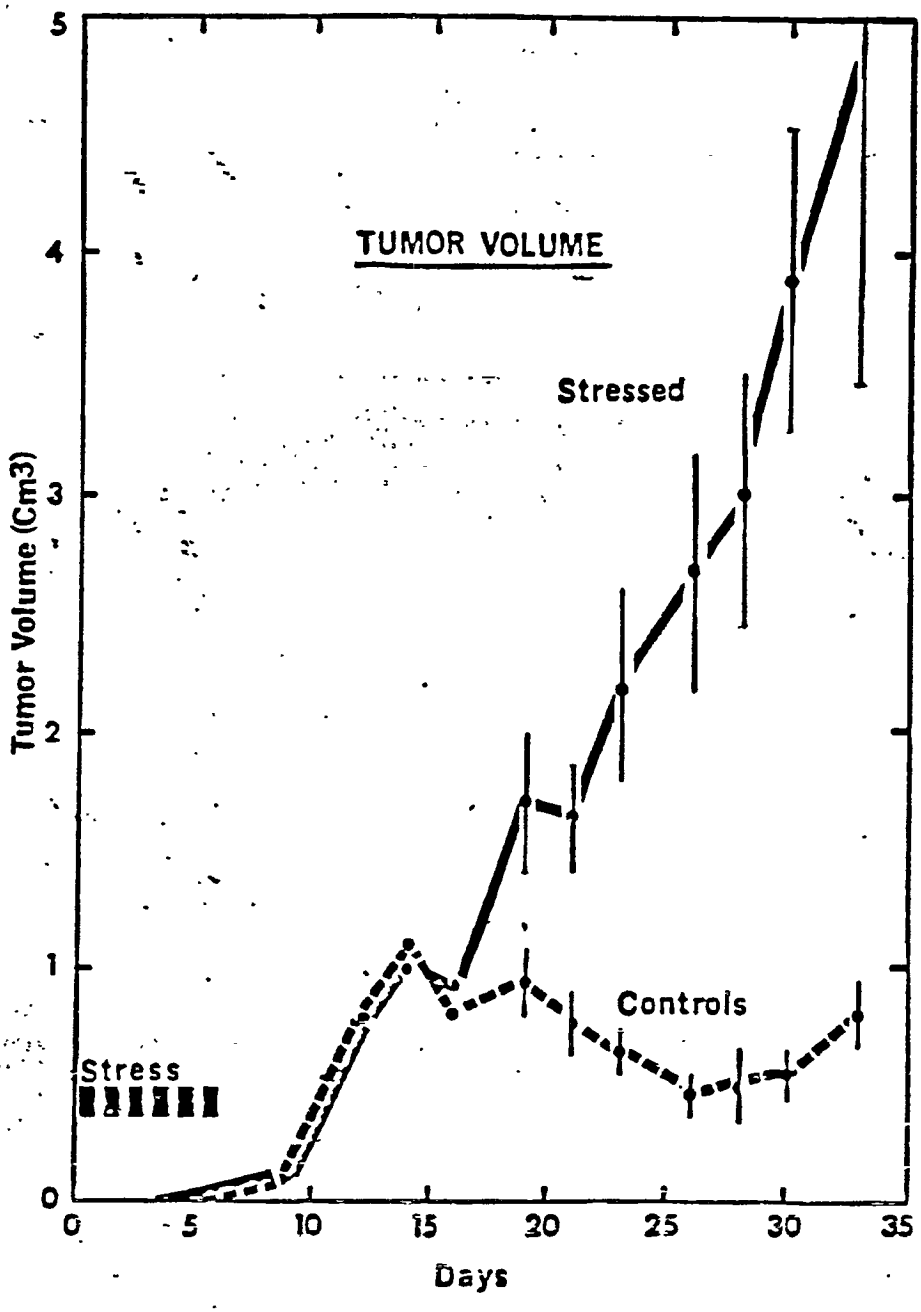

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Figure 7. Stress-associated influences on tumor growth in a host exposed to an intermittent course of mild anxiety stress. The stress was induced by slow rotation at 45 rpm for ten minutes out of each hour during the first six days following tumor implantation. The tumor was the 6C3HED ascites lymphosarcoma inoculated subcutaneously into C3H/He female mice about ten weeks old. Food and water were available at all times during the course of the experiment.

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Effects of Stress on Thymus Involution.

Figure 8 illustrates and compares the influence of three distinct stress factors on the induction of thymus involution. Figure 8a shows the effect of the LDH virus on the loss of thymus weight as a function of time following infection, whereas Figure 8b indicates that a similar effect can be induced by the administration of a single dose of dexamethasone. In Figure 8c similar effects were obtained by the induction of anxiety stress through the slow rotation of mice at 45 rpm.

Rotational Stress and Amino Acids.

Figure 9 illustrates some of the physiological consequences of a mild rotation-induced anxiety stress (31a), as indicated by significant alterations in the amino acid profile of the urine of normal mice compared with analogous stressed animals. Some of the more conspicuous changes which may be noted are the decreased concentration of lysine, and in contrast, the elevations of glutamic acid, alanine, valine, leucine, tryptophan, phenylalanine, tyrosine, and isoleucine.

These observations demonstrate the sensitivity of the collocated, concentration-prioritized amino acid profiles for detecting subtle physiological changes (43). Of equal importance, the combined data illustrate the remarkable sensitivity of the organism in responding to both its internal and external environments.

Although there is no direct experimental evidence that the observed stress-induced changes in amino acid concentrations are related to the observed tumor growth enhancement, it has been reported that changes in certain amino acid levels have been associated with a reduction of immunological competence (21).

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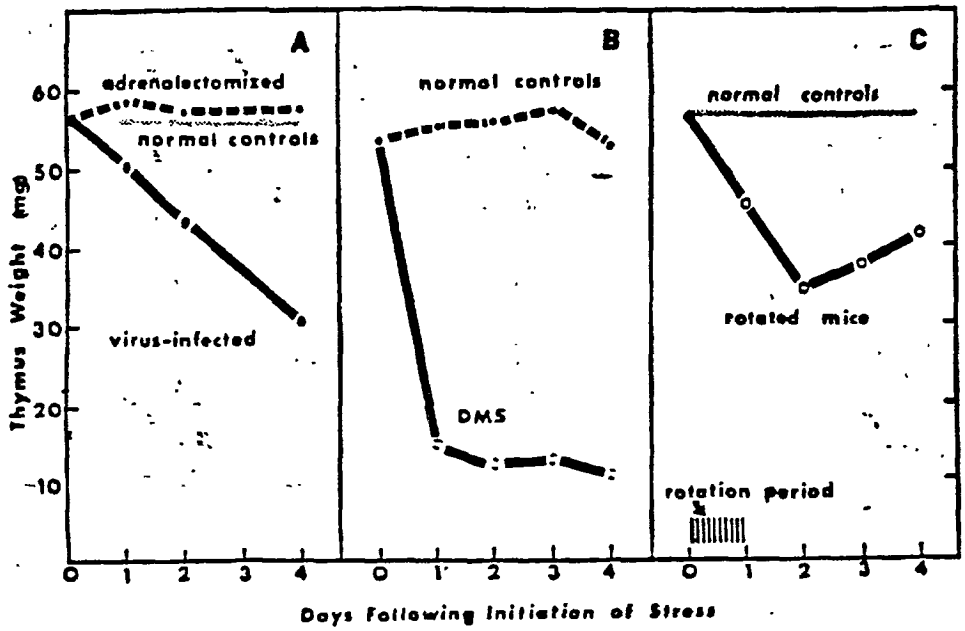

Figure 8 . Thymus involution variously produced by an infectious agent (the LDH-virus), a biochemical substance (DMS, a synthetic corticoid), and anxiety-stress associated with the disorienting effects of slow rotation of an entire cage of mice following their removal from protective storage.

(A) The LDH-virus was injected on day 0 into both intact and adrenalectomized female BAF mice; as shown by the left-hand panel, the thymus involution in the intact mice contrasts with the lack of response of the adrenalectomized animals.

(B) The thymus involution produced by a single injection of an insoluble suspension of DMS, which was absorbed over an extended period, is shown in the middle panel.

(C) The thymus destruction resulting from stress associated with cage rotation at 45 rpm for 10 minutes out of each hour for a 24-hour period is shown in the right-hand panel. During this mild stress period the plasma corticosterone was elevated approximately 5-fold.

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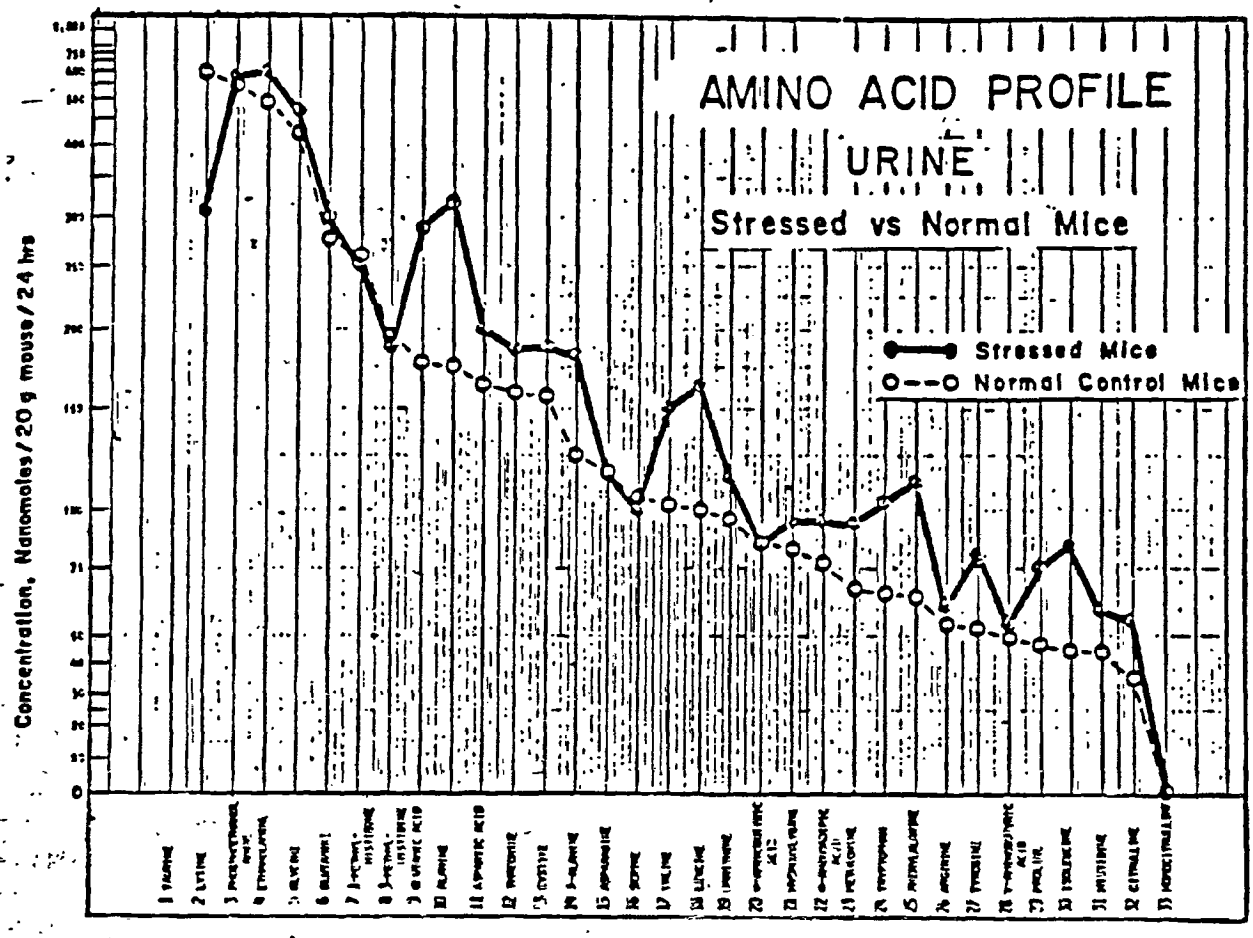

Figure 9, Amino acid concentration profiles of the urine of mildly stressed mice as compared with normal quiescent control animals. The anxiety stress was quantitatively induced under highly controlled experimental conditions using a slow-speed rotational device at 45 rpm intermittently for 24 hours. The urine was a 24-hour sample collected during the rotation period. The rotational intermittence was controlled automatically and was programmed for ten minutes rotation and fifty minutes rest each hour. Food and water were available at all times during the 24-hour period. The centrifugal force of the rotation was less than one G, which permitted the mice to move about their cage at will.

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Simulated "Stress" by a Synthetic Corticoid.

Figure 10 provides further experimental evidence concerning the effects of stress on neoplastic behavior. In this experiment the neuroendocrine pathways were intentionally bypassed and physiological "stress" was simulated by the introduction into the animals of a highly potent synthetic corticoid. In this case, a single injection of fluocinolone acetonide (FCA), which is closely related chemically to dexamethasone, was administered at various times following transplantation of the Gardner lymphosarcoma. When this corticoid was administered seven days following implantation of the tumor, no tumor regressions occurred, in contrast to 70% regressions observed in the untreated control C3H/He mice. However, this corticoid effect was systematically diminished when the compound was administered at later times in the course of tumor growth. These data may be interpreted as a demonstration of the damage inflicted upon the host immunological apparatus by glucocorticoids, whether endogenous or administered. The expression of this damage by a decrease in tumor regressions, however, is dependent upon the stage of tumor development at the time of glucocorticoid administration. This is indicated by Figure 11, which shows the tumor growth enhancement resulting from FCA injections at the various times following tumor implantation. In this experiment, maximum tumor growth enhancement occurred following FCA administration 7 days after implantation. Lesser effects were observed when the compound was injected on the fourteenth and twenty-first day post-implantation, while the FCA had no effect on tumor growth at 28 days after implantation.

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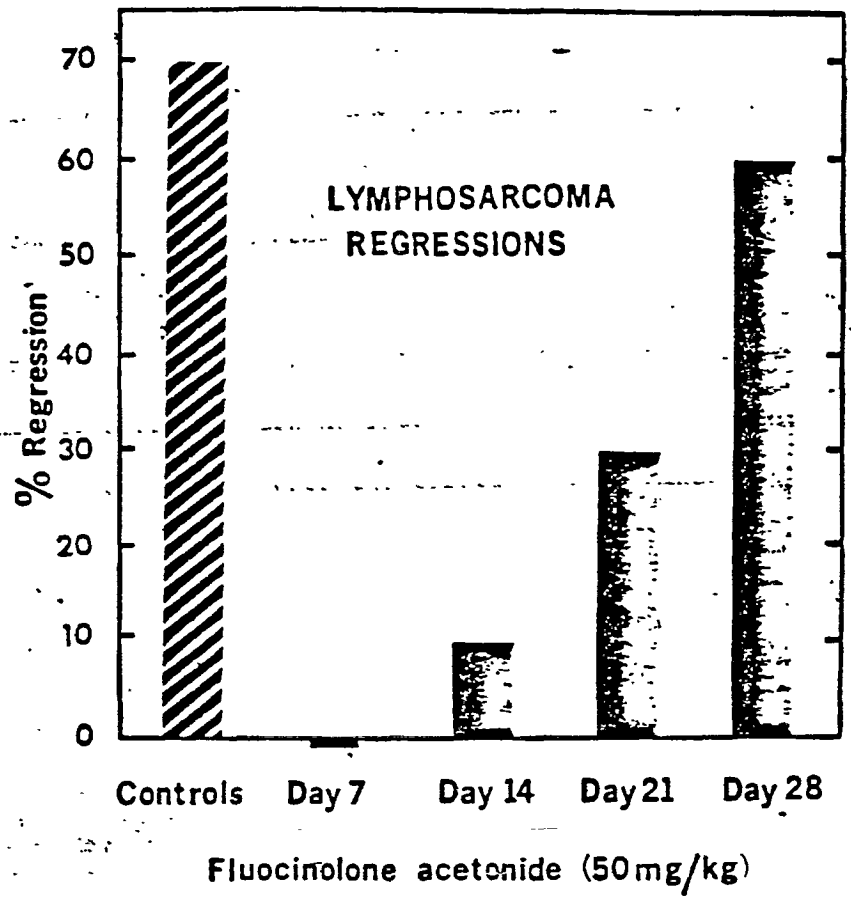

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Figure 10. The influence of a synthetic glucocorticoid in inhibiting the regression of established tumors. The synthetic glucocorticoid was fluocinolone acetonide (FCA) and was injected intraperitoneally at a dose of 50 mg/kg suspended in physiological saline, at the times indicated following tumor implantation. The tumor was the Gardner lymphosarcoma (6C3HED), implanted subcutaneously in the hip of C3H/He female mice. Ten mice were used for each group.

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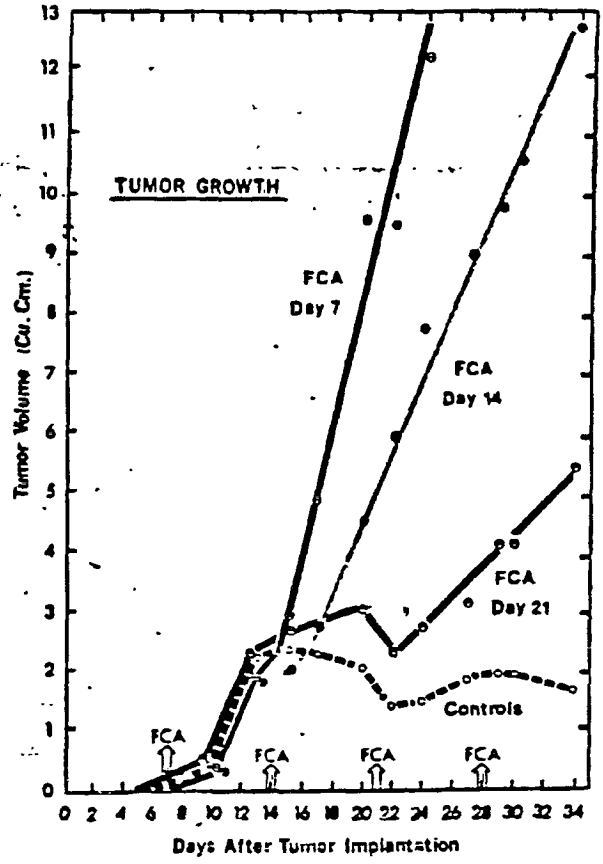

Figure 11. Different tumor growth rates resulting from the injection of a synthetic corticosteroid at various times in respect to tumor implantation. The groups of mice were the same as those shown in Figure 10. Those mice receiving FCA on the 28th day post-implantation exhibited a tumor growth rate that was indistinguishable from that of the untreated tumor-bearing controls.

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Population Density Experiments.

It is important for the investigator to know what effects various numbers of animals housed in a standard cage will have upon the results of an experiment. In order to minimize animal fighting, or for other reasons, some investigators house their mice or rats one per cage, while others engaged in similar studies may have various numbers ranging from two to ten per cage.

Several publications have described the stressful effects of "cage-crowding", and the alleged consequential influences on the growth or incidence of various tumors, or other disease processes, presumedly due to alterations in the immunological competence of the hosts (5,6,13,15). The adverse psychosocial effects associated with isolation-rearing in contrast to standard rearing have also been reported (1,18-20, 23,24,35).

We have undertaken experiments to re-examine this question utilizing protective, minimal stress facilities and low-stress handling techniques, in order to control a maximum of the known forms of stress in carrying out experiments concerned with various biological processes. Experiments were designed to determine the different levels of anxiety stress among mice caged in groups of 1, 2, 3, 5, 10, 15, or 20, and held for relatively long periods in standard plastic cages. There were no mice of the opposite sex either in the cages or in the immediate vicinity, and thus there were no obvious pheromones which could provide uncontrolled stress-inducing circumstances.

Effects of Population Density upon Plasma Corticosterone Levels.

It has been established in these studies that various population densities of caged female mice do not constitute, in themselves, a basis for "cage crowding stress". This was determined by a failure to detect differential levels of plasma corticosterone (CSR) in mice caged either 1, 2, 3, 5, 10, 15, or 20 per standard plastic cage. It was expected, however, that quite

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different effects would be observed when employing competitive male mice under analogous experimental circumstances. It was thus surprising to observe that various cage population densities of males also failed to demonstrate elevated plasma corticosterone. Figure 12 illustrates the similarity of plasma corticosterone levels in male C3H/He mice when housed in various population densities, as compared with females under similar circumstances.

Effects of Population Density upon Relative Food Consumption.

In addition to the CSR data, Figure 12 also shows that the differences in food consumption observed with females maintained under conditions of various population densities, also applies to male mice. These data also show an unexpected systematic difference in individual daily food consumption, which is expressed as a concave curvilinear function of population density. Mouse weight was taken into consideration in the plotting of these data.

Our interpretation of these observations is that the low-stress animal facilities, in conjunction with our experimental design, have permitted a sensitive quantitative detection of the homeostatic thermal regulatory mechanism that controls caloric intake as a function of body temperature maintenance requirements.

It is obvious that body heat-loss occurs more rapidly in mice residing alone, or in small numbers, as compared with the loss in high density populations where huddling and multiple body contact occurs. Other possible explanations for these observations have been considered and experimentally examined; however, the constant body weight data shown in Figure 13 and the absence of hierarchial-imposed weight differences are all consistent with the caloric requirement hypothesis.

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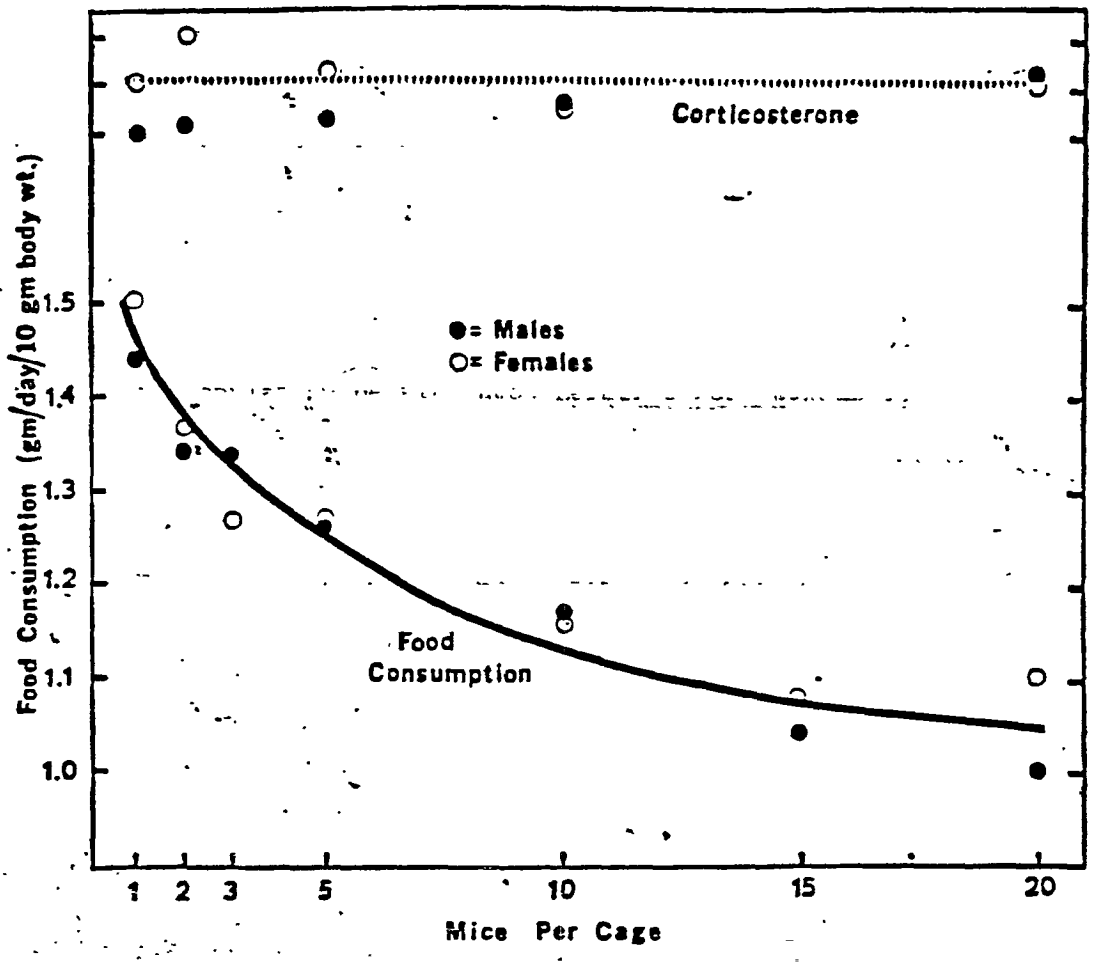

Figure 12 Comparison of the effects of population density upon the food consumption and daytime plasma corticosterone levels of male and female mice. Food consumption is plotted per 10 grams of body weight, to correct for body weight differences in male and female mice. Food consumption was determined by weighing the food 3 times per week; each food consumption point represents the mean over a four week period. Corticosterone levels were determined at the end of the experiments. Each corticosterone point represents a plasma pool from five mice. C3H/He mice were employed.

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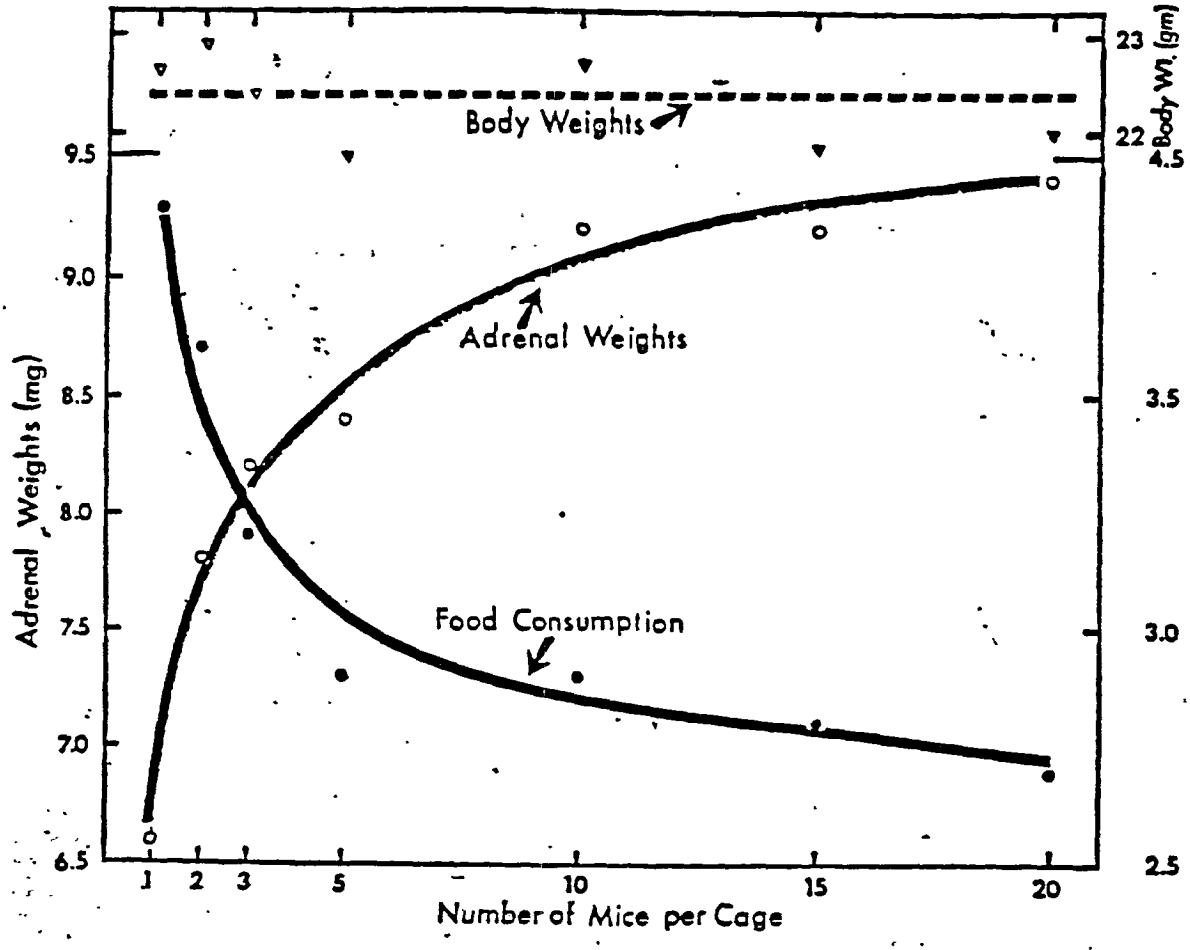

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Figure 13. Effects of population density upon food consumption, body weights and adrenal weights of C3H/He female mice. When calculated by the chi-square method, the combined adrenal weight data of 1, 2, and 3 mice per cage (32 animals) compared with 10, 15, and 20 mice per cage (45 animals) gave a $P < 0.05$.

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Irrespective of the mechanism, these observations are relevant to the experimental design of nutritional, neoplastic, and related experiments in which caloric intake is of importance. In this respect, it is appropriate to recall the influences that have been reported, of varying caloric intakes (33,45,46) and temperature conditions (9,49,50) upon carcinogenic processes.

Effects of Population Density on Adrenal Weights.

In contrast to the absence of any influence of cage population density upon plasma corticosterone, there does seem to be a systematic increase in adrenal weight as a function of the number of animals per cage. This is shown in Figure 13. We have considered several hypotheses to rationalize these findings. One possibility is that changes in adrenal weight under these circumstances may be a more sensitive parameter of stress than plasma corticosterone levels. However, based upon previous experiments, this seems somewhat unlikely. Another possibility that we have considered is that the increase in adrenal weight is an expression of adrenal medulla hyperplasia rather than adrenal cortex hyperplasia. The adrenal cortex is, of course, the tissue involved in the production of corticosterone, whereas the adrenal medulla is concerned with the production of catecholamines, the most conspicuous being epinephrine and norepinephrine.

Dr. James Henry, a "stress physiologist" at the University of California, has evidence that the stress function can be divided into two separate categories, with anxiety-stress being primarily associated with the adrenal cortex, whereas stress involving fright or fury as in fighting animals, or in the establishment of social hierarchy, involves the adrenal medulla and its

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catecholamines, rather than, or in addition to, the adrenal cortex and glucocorticoid elevation (10). Histological examination of the adrenals is obviously required to determine which elements show either cellular depletion or tissue hyperplasia. Another possible explanation is that the day-time plasma samples which were employed do not accurately reflect the relative CSR levels at night during the competitive feeding period.

Effects of Population Density upon Tumor Growth.

A part of the testing procedure on possible effects of cage crowding, included monitoring the animals for tumor incidence, tumor latent periods, tumor growth rates, and survival times following the implantation of various transplantable tumors. One of the tumor types that was reported to be responsive to "cage crowding" was a transplantable B-16 mouse melanoma (5, 6).

On day 29, following initiation of a population density experiment involving 1, 2, 3, 5, 10, 15 or 20 mice per cage, all of the BDF female mice that were in the experiment were implanted with the B-16 melanoma. Tumor measurements were carried out twice a week in order to determine relative tumor growth rates. Tumor latent periods were also determined, as well as the survival times of the hosts.

In contrast to the previous reports of Dechambre and Gosse (5, 6), we found that there was no significant influence on the tumor growth rate, or the survival times of the hosts, as a function of the number of animals per cage. Thus, the behavior of this particular tumor was essentially the same in those animals that were housed one to a cage as those mice that were housed 20 to a cage, as well as in the other intermediate population densities.

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In contrast to this observation of a lack of effect of cage population density upon the B-16 melanoma, Figure 14, nevertheless, shows the effects of population density upon the growth of the Gardner 6C3HED lymphosarcoma, as indicated by differences in the maximum tumor volumes that were attained in the various groups. This illustration also shows the effects of population density upon food consumption prior to tumor implantation. It is unclear whether the effects of population density upon the growth of this tumor may be related to the earlier differences in food consumption, or to the observed differences in adrenal size shown in Figure 13, or other factors.

Effects of Population Density upon Tumor Regressions.

Since tumor regression is a good index of immune competence, we used that parameter as one means for determining the optimal population of mice per cage in respect to this parameter. The results of these experiments were unexpected. The tumor regression rate of the Gardner 6C3HED lymphosarcoma in C3H/He female mice where there was only one animal per cage, was 60 percent. This was in contrast to other population densities of 2, 3, 5, 10, 15, or 20 mice per cage, which had an average of 93 percent regressions, with a range of 80 to 100 percent ($P < .001$). Thus, based upon ability of female mice to reject a tumor challenge, all tested densities of group housing were preferable to that of single animal isolation. See Table 1. As shown in Table 2, male C3H/He mice housed one or two per cage had a lesser ability to reject the Gardner 6C3HED lymphosarcoma than did those housed 3, 5, 10, 15 or 20 per cage. Unlike female mice, male mice housed 2

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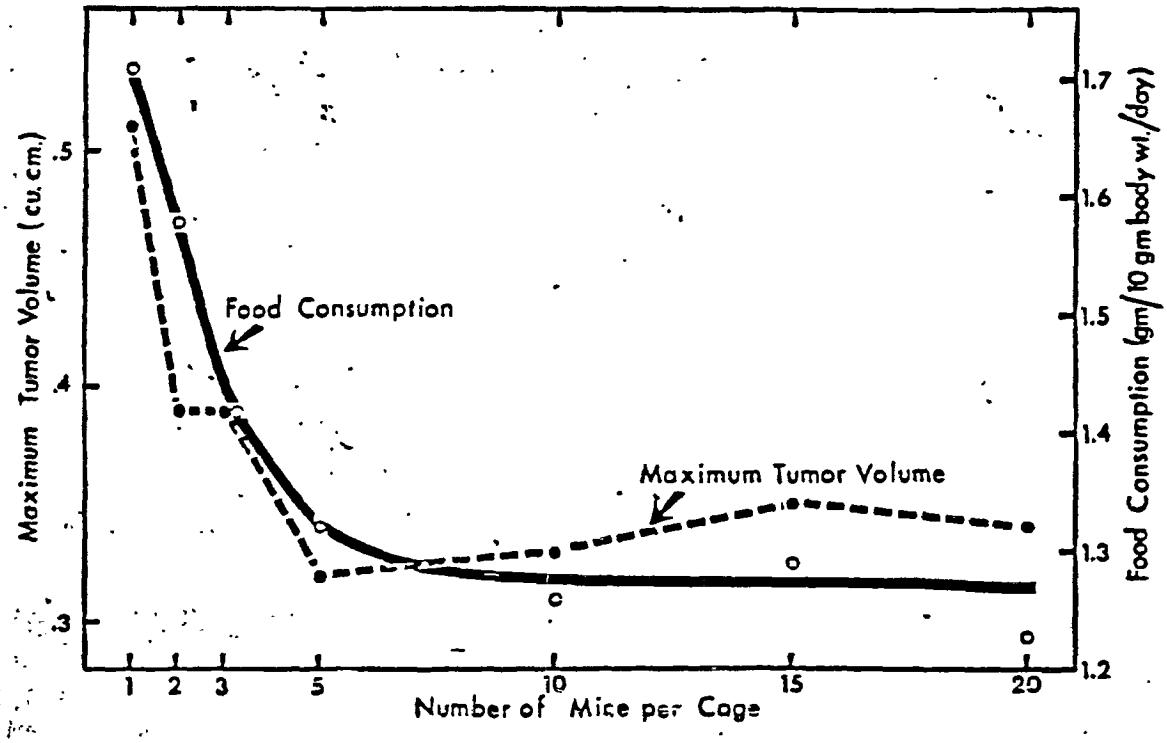

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Figure 14. Effects of population density upon food consumption and subsequent maximum tumor volumes in female C3H/He mice. Each food consumption point represents the mean of determinations made at 3 or 4 day intervals during the period from 3 to 36 days following the initiation of the various population densities. Subsequently, on day 56 of the experiment, all mice were implanted with the Gardner lymphosarcoma.

Source: https://www.industrydocuments.ucsf.edu/docs/xtwm0000

TABLE 1.

EFFECTS OF POPULATION DENSITY UPON TUMOR REGRESSIONS IN FEMALE MICE

No. per Cage	Percent Tumor Regressions[a]	
1	60%	
2, 3, 5, 10, 15, or 20	93%	P<.001[b]

a) Tumor regressions were determined on day 25 after tumor implantation. The Gardner 6C3HED lymphosarcoma was implanted into C3H/He female mice, 56 days after initiation of the various population densities.

b) P value was determined by the chi square method, comparing the data from 1 mouse/cage with the combined data from 2, 3, 5, 10, 15, or 20 per cage.

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TABLE 2.

EFFECTS OF POPULATION DENSITY UPON TUMOR REGRESSIONS IN MALE MICE

No. per cage	Percent tumor regressions[a]	
1	70%	
2	65%	
1 or 2 (combined data)	67.5%	
3, 5, 10, 15, or 19 (combined data)	84.6%	P<0.01[b]

a) Tumor regressions were determined on Day 25 after tumor implantation. The Gardner 6C3HED lymphosarcoma was implanted into C3H/He male mice, 56 days after initiation of the various population densities.

b) P value was determined by the chi square method, comparing the combined data from 1 or 2 per cage with the combined data from 3, 5, 10, 15, or 19 per cage.

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per cage exhibited no advantage over isolated mice, with respect to tumor growth. It therefore appears that male mice should not be housed one or two per cage. However, any number over 2 per cage, up to 20 per cage, yielded similar results; no optimum number per cage in this range was exhibited. Despite the differences in tumor regressions, the effects of housing male mice 1 or 2 per cage had no observable influence upon daytime plasma corticosterone levels. It was noted, however, that male mice housed 2 per cage exhibited lower food consumption and somewhat lighter body weights than those housed 1 or 3 per cage.

These and related data demonstrate the importance of recognizing and controlling experimental circumstances that produce stress, and its inevitable accompaniment of immunological impairment, which in turn, may alter tumor growth rates and subsequent tumor regressions (11, 22, 40, 41).

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Source: https://www.industrydocuments.ucsf.edu/docs/xtwm0000

Effects of Hanging, Wire-Bottom Cages upon the Stress and Health Status of Experimental Rodents.

Most investigators do not have complete control over the animal facilities which they are required to use for their biological experiments. This may not be a serious handicap if the facilities were designed and constructed employing the latest state of the art. Regrettably, this is not usually the case. As an example, some facilities provide expensive metal racks which contain hanging, wire-bottom cages for holding rats or mice. Such animal cages are designed primarily for the convenience of animal caretakers since the excreted feces and urine fall through the wire and are periodically removed by various expeditious procedures. Unfortunately, both the animal inhabitants and conscientious investigators pay a high price for this convenient but unscientific arrangement. The evidence is as follows:

Female mice housed in wire-bottom cages, mounted within low-stress facilities, exhibited a 10 percent decrease in food consumption and a 30 percent decrease in water consumption, as compared with the food and water consumption of similar mice housed in standard plastic cages containing corn-cob bedding. Food and water consumption was measured twice weekly for 8 weeks. Each experimental group consisted of 50 C3H/Bi female mice housed 10 per cage.

As shown in Figure 15, circulating corticosterone was twice as high in the plasma of the mice residing in wire-bottom cages as in analogous mice housed in standard plactic cages with corn-cob bedding. This high hormone level is indicative of chronic stress, which has far-reaching effects upon the physiological state of the animals. This hormonal elevation, in turn, raises questions concerning the validity of otherwise carefully designed experiments, employing wire-bottom cages for animal housing.

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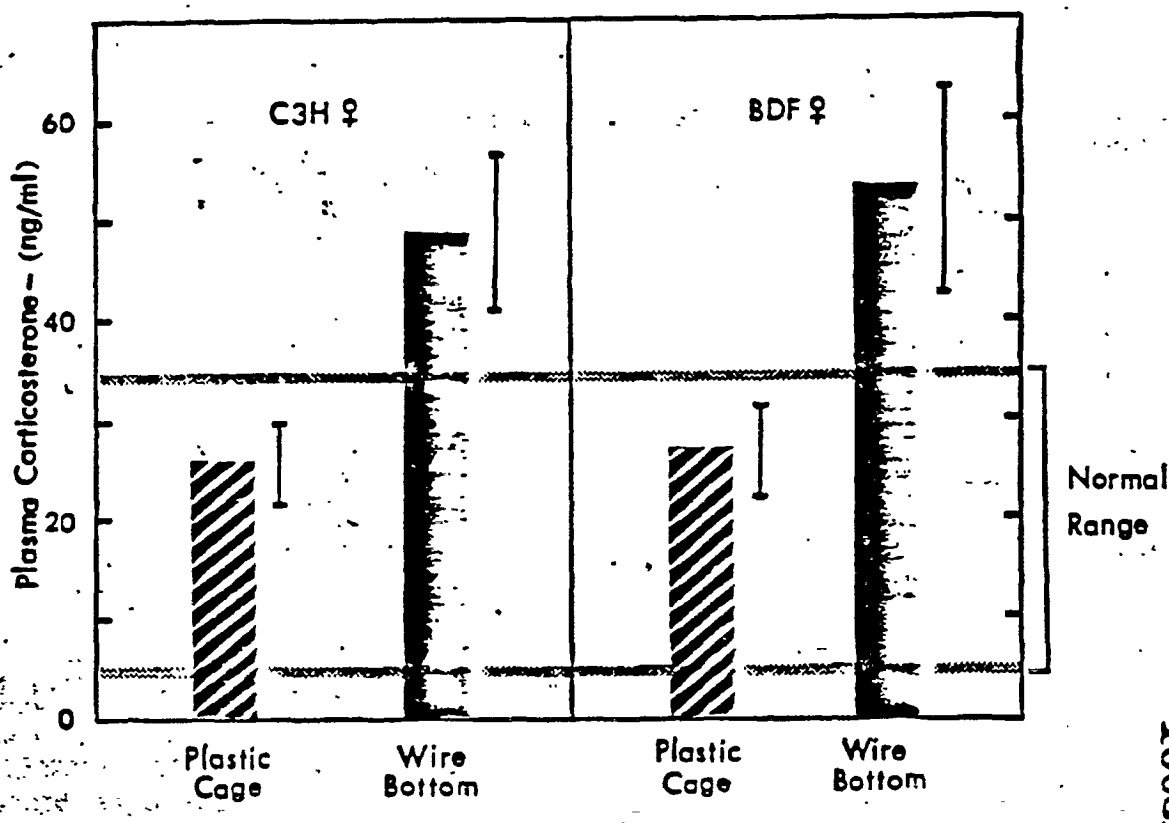

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Figure 15. Comparison of the effects of hanging wire-bottom cages versus plastic cages with bedding upon daytime plasma corticosterone levels as found in two strains of female mice.

Source: https://www.industrydocuments.ucsf.edu/docs/xtwm0000

In addition to, or perhaps as a result of the cage-induced stress with its various physiological ramifications, there was a substantial difference in animal survival. For example, as indicated in Figure 16, at 14 months of age, 64 percent of the mice in the wire-bottom cages were dead. This is in comparison with 14 percent deaths in analogous mice maintained in plastic cages with bedding ($P < .0001$). These death rates are a reflection of the comparative health status of the two groups. In a separate experiment, a statistically significant adrenal enlargement ($P < .01$) was observed in female mice held in wire-bottom cages for 50 days as compared to analogous mice housed in plastic cages for the same period of time. The results of these and related studies indicate that hanging wire-bottom cages should not be used for serious animal experimentation.

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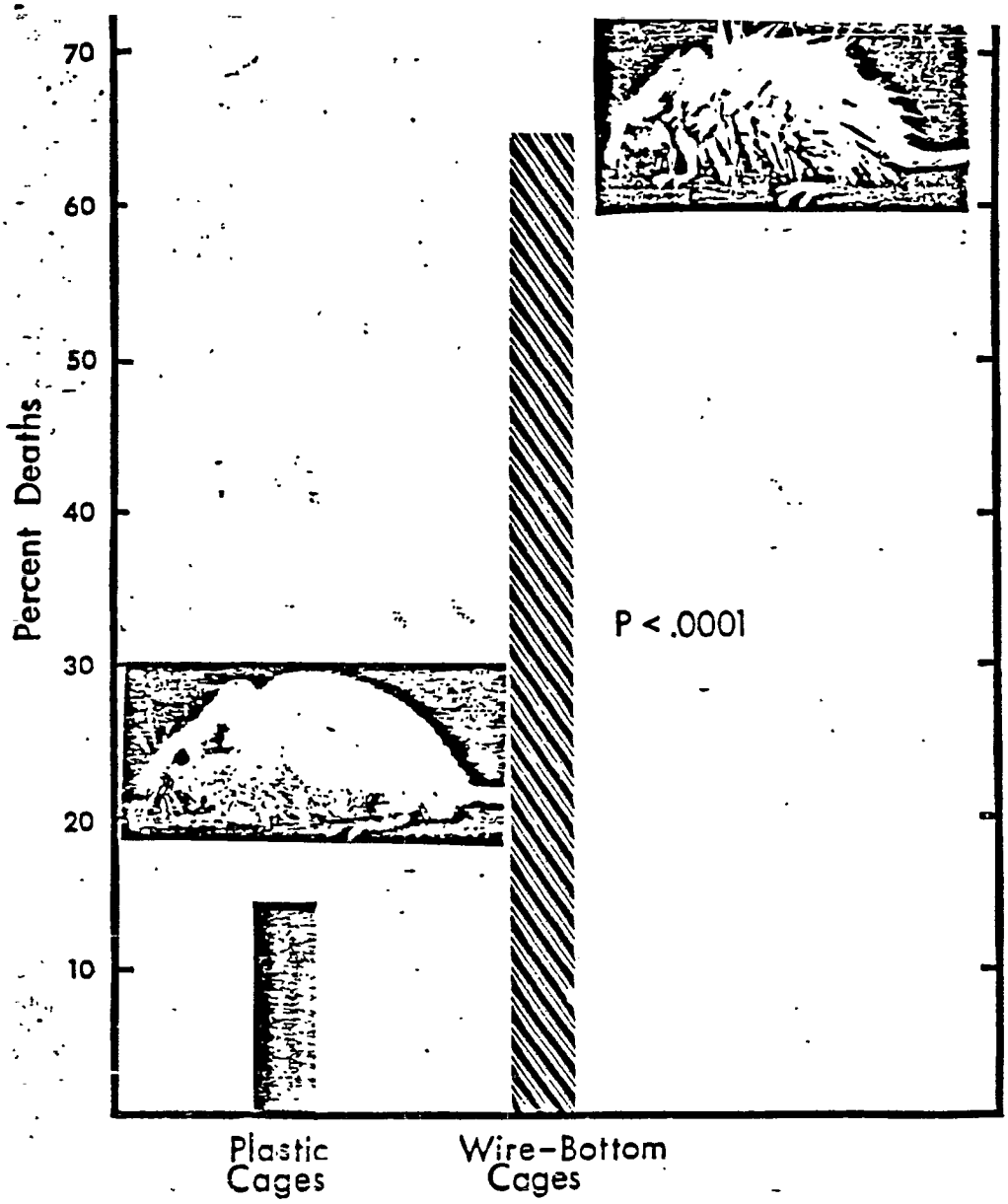

Figure 16. Comparative deaths in C3H/Bi female mice housed in standard plastic cages with bedding, and those housed in standard hanging wire-bottom cages. Each group consisted of 50 mice, housed 10 per cage; the observation period for both groups was 14 months. The probability value was determined by the chi-square method.

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Stress Effects of Proximity to Opposite Sex. We have demonstrated that mice that are being bled or injected, or otherwise handled in ways to generate anxiety stress, are able to communicate their stress to other mice in the same room, as evidenced by the finding of elevated plasma corticosterone levels in the untouched animals following such procedures.

Figure 17 shows the results of a study intended to provide additional information about stress communication among mice. Combinations of cages of male and female mice were positioned on the shelves of our protective housing facilities, where the air flow was uni-directional and constant along the shelves.

These studies, termed "Upwind/Downwind", were initiated to determine whether pheromones play an important role in stress communication in mice, and whether airflow along the shelf on which the mice were housed was the primary vector in stress communication. The data suggest that air-borne pheromones are either not involved, or at least are not the only source of stress communication. All of the female mice and many of the male mice showed significant stress-elevated corticosterone levels, and such elevations were not always related to being upwind or downwind of mice of the opposite sex. Several vertically adjacent housing shelves were used in this experiment and the stress seems to have been communicated, especially in the case of female mice responding to the presence of male mice located on vertically adjacent shelves.

This work needs to be repeated for verification; also additional experiments of a similar nature should be conducted in which the shelves of mice are physically separated vertically from each other for varying distances in order to further test the significance of subsonic, supersonic, or audible sound as the primary factor in stress communication, as compared with pheromones (12). The existence of stress communication is extremely important in the conduct of experimental procedures with animals, and in establishing the optimal physical design of animal housing facilities.

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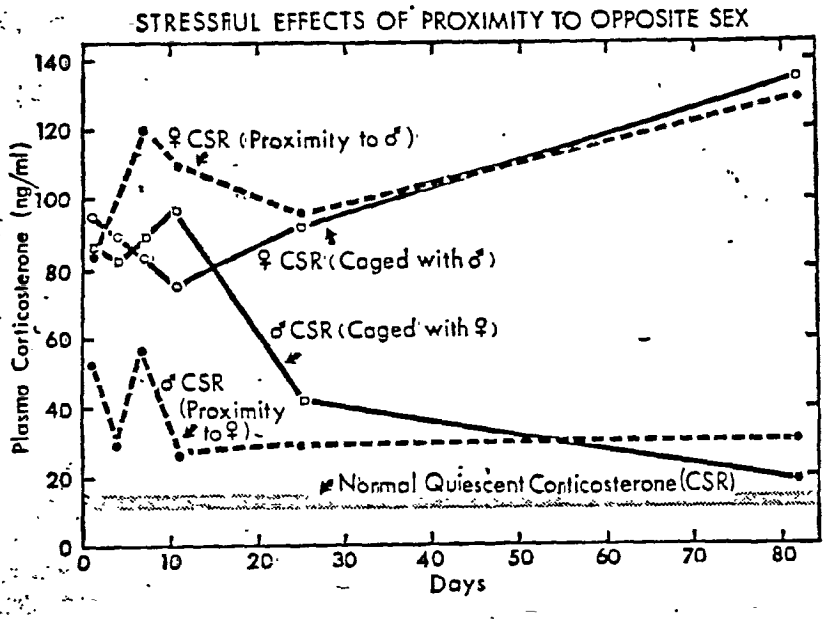

Figure 17. Plasma corticosterone levels of mice caged with the opposite sex compared with mice caged in proximity to separate cages containing mice of the opposite sex or sexually active males and females caged together. Female corticosterone values were obtained from non-pregnant individuals only. The "normal quiescent" levels were obtained in the complete absence of the opposite sex. All mice were housed 5 per cage in standard plastic cages with corn-cobb bedding.

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Concentration of Stress Hormone (Corticosterone) in the Blood of Male and Female Mice Housed in Various Proximities to Each Other. Most experimental animal facilities house male and female animals in the same room, frequently on the same shelf, or, for some purposes, in the same cage. While various physiological and stressful consequences of caging males and females together have been known or suspected, it is essential to have specific qualitative and quantitative information on the precise stress status resulting from male-female proximity in order to evaluate the changes that may occur regarding immunocompetence or other important physiological states. Since an elevated level of circulating corticosterone in the blood is a reliable biochemical reflection of stress, and a useful index for predicting thymus damage, destruction of protective T cells, and consequential impairment of immunological competence, we measured this hormone in both male and female mice under various conditions of proximity to the opposite sex.

Male Mice. As shown in Figure 17, male mice housed in the same cage with females exhibited an average of a 4-fold plasma corticosterone elevation during the early exposure period (days 1 through 11) following the introduction of females. This increase was in comparison with "quiescent" males that were housed in a separate unit in the complete absence of females. Males caged without females, but in close proximity to other cages holding sexually active males and females, exhibited a 3-fold plasma elevation on exposure days 1 through 7. Such proximity-affected corticosterone levels were compared with those of males housed in separate units. By exposure day 25, however, males caged with females, as well as those caged in proximity to sexually active males and females, exhibited a drop in corticosterone to approximately 2 times the quiescent level. This low level was maintained through exposure day 82 in the males housed in separate cages but in proximity to the sexually active mice. In sexually intact males housed in the same cage with females,

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the corticosterone level by exposure day 82 was essentially at the quiescent level.

Female Mice. Females caged with males exhibited about a 4-fold plasma corticosterone elevation on exposure days 1 through 11, while females caged seperately but in close proximity to sexually active males and females, also showed about a 5-fold elevation during this initial time period. By exposure day 25, both groups of females had maintained a 4 to 5-fold corticosterone elevation, compared with quiescent females. However, instead of gradually decreasing with exposure time as did the males, this high corticosterone level continued to increase to a 7-fold elevation by exposure day 82. This occurred with the non-pregnant females caged with males as well as with those females caged in proximity to cages of sexually active males and females.

The most unexpected finding of the male-female proximity studies was that the exciting or disturbing influences of the two sexes upon each other were not restricted to male-female cage mates, but also were observed when the two sexes were caged separately but in the same vicinity. Initial observations tentatively assigned these distant influences to airborne pheromones, potent hormone-like sustances that are excreted by insects and animals as sex attractants. However, the airflow in our animal facilities is carefully controlled, providing a unidirectional flow through the enclosed shelves. It is then exhausted to the exterior, so that no pheromone-contaminated air is recirculated following exposure to the mice and their effluents. Since the stress transmissions of the male-female effects were about the same both upwind and downwind on the same shelves, as well as vertically to the enclosed shelves both above and below the test animals, an exclusive effect of pheromone action seems unlikely. This raises the question of other possible means of stress communication, such as ultrasonic

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signals (48), that may be functioning to explain the transmission of male-female exciting influences. Irrespective of the mysteries associated with these observations, it is relevant for investigators to be aware of these potent near and far proximity effects that are capable of altering various physiological parameters, including host immunocompetence.

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DISCUSSION AND IMPLICATIONS

Corticosterone Levels.

As a consequence of several interrelated developments, it has been established that experiments using mouse models for the study of immunological and neoplastic problems are undoubtedly complicated, and in some cases compromised, by the inadvertent and unappreciated stress that is present in mice maintained in conventional housing facilities and handled in the usual manner. The anxiety-stress of such mice is readily demonstrated by a determination of their plasma corticosterone. The quiescent corticosterone level of unstressed animals in our protective animal rooms is 0 - 35 ng/ml during the morning hours, whereas in mice housed in conventional animal rooms, the plasma corticosterone levels are usually elevated and have been shown to be in the range of 150 - 500 ng/ml for those tested.

Stress-Induced Cellular and Tissue Lysis.

When such glucocorticoids are present in the blood at concentrations above normal, they have lytic effect on lymphocytes and upon lymphatic tissues such as the thymus, nodes and spleen. Since these cellular elements are important components in immunological defense, the potential consequences of their depletion on latent pathological processes seem obvious. There thus appears to be a logical sequence of biochemical and cellular events that lead from stressful stimuli to the enhancement of various pathological processes, including that of cancer.

However, not all neoplastic processes exhibit enhancement following exposure of the host to stress. Rapidly growing tumors that are syngeneic with their host have not responded to the moderate stress which has been effective with

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slowly growing tumors that are partially under host control. These observations suggest that stress may have potentiating effects only on those malignancies that are usually under control of the host's defense mechanisms, such as the 6C3HED lymphosarcoma when grown in C3H/He mice.

Population Density.

It is important for the investigator to know the caging conditions that are optimum for his experimental requirements; or, more specifically, to know what effects various numbers of animals per standard cage will have upon the results of an experiment. In order to minimize animal fighting, or other unwanted animal interaction, some investigators house their mice or rats one per cage, while other laboratories engaged in similar studies may have various numbers, ranging from two to ten, per cage. We have carried out a series of experiments to examine this question in some depth. A useful approach is to employ an appropriate tumor-host relationship as a sensitive means for determining the immunological status of experimental animals as a function of population density. Since the host-induction of tumor regression is a good index of immune competence, that parameter was employed as one of several means for determining the optimal cage population.

The results of these experiments were unexpected. The tumor regression rate of the 6C3HED Gardner lymphosarcome where there was only one animal per cage was 60 percent. This was in contrast to the other population densities of 2, 3, 5, 10, 15, or 20 mice per cage, which had an average of 93 percent regressions, with a range of 80 to 100 percent ($P < .001$). Thus, based upon host ability to reject a tumor challenge, all tested densities of group housing were preferable to that of single animal isolation. When the maximum

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tumor volume attained was employed as an indicator of immunological competence, the greatest 6C3HED tumor growth was observed in the isolated female mice, held one per cage, while the smallest tumors were found in the female mice housed five per cage ($P < .005$). However, when the same tumor was implanted into male C3H/He mice, the greatest tumor growth was observed in those housed two per cage, and no clear-cut optimum number per cage, in the case of male mice, was determined. It was concluded that both male and female mice were stressed by isolation, and in addition, male mice housed two per cage exhibited the effects of greater stress.

In contrast to the results obtained using the 6C3HED lymphosarcoma, no effects of cage crowding on the B-16 tumor were observed, presumedly because of the lack of host immunological control over this rapidly growing pigmented tumor.

These data demonstrate the importance of recognizing and controlling experimental circumstances that produce stress and its inevitable accompaniment of immunological impairment, which in turn, may alter the growth rate and subsequent tumor regression of sensitive tumors.

Effects of Wire-Bottom Cages.

Circulating corticosterone is twice as high in the blood plasma of mice residing in wire-bottom cages as in analogous mice housed in standard plastic cages with corncob bedding. This high hormone level is indicative of chronic stress, which has readily measurable effects upon the physiological state of the animals. These biochemical defects, in turn, raise questions about the validity of otherwise carefully designed experiments.

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There is also a substantial difference of animal survival in wire-bottom cages. For example, at 16 months of age, 66 percent of the mice housed in wire-bottom cages were dead; this is in comparison with 16 percent deaths in analogous mice maintained in plastic cages with bedding. Such death rates are probably a reflection of the comparative health status of the two experimental groups. It is such unappreciated environmental differences that cause some of the contradictions among data obtained in various laboratories. The results of these and related studies indicate that hanging wire-bottom cages should not be used for serious animal experimentation.

Plasma Corticosterone in Male and Female Mice in Various Proximities to Each Other. Many experimental animal facilities house male and female animals in the same room, frequently on the same shelf, or, for some purposes, in the same cage. While various physiological and biochemical consequences of caging males and females together have been known or suspected, it is essential to have specific qualitative and quantitative information on the precise stress status resulting from male-female proximity, in order to evaluate the changes that may occur in immunocompetence or other important physiological states. Since the level of circulating corticosterone in the blood is a useful biochemical measurement of stress, and an index for predicting thymus damage, destruction of T cells, and a consequential impairment of immunological competence, this hormone was measured in both male and female mice under various conditions of proximity.

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When the stress hormone levels are measured in male mice caged together with females, a 4 to 6 fold elevation of corticosterone was found, as compared with quiescent males caged in a separate room. Essentially the same increase in stress hormone was found in females that were housed in the same cage with males. The most unexpected finding was that these exciting or disturbing influences that the two sexes exert upon each other were not restricted to male-female cage mates, but also were observed when the two sexes were caged separately but in proximity to cages holding sexually active males and females.

Initial observations tentatively assigned these distant influences to airborne pheromones, potent hormone-like substances excreted by insects and other animals as sex attractants. However, the airflow in our animal facilities is carefully controlled, providing a unidirectional laminar flow through the enclosed shelves. It is then exhausted to the exterior, so that no pheromone-contaminated air is recirculated following exposure to the mice and their effluents. Since the stress transmissions of the male-female effects were about the same both upwind and downwind on the same shelves, as well as vertically to the enclosed shelves both above and below, an exclusive effect of pheromone action seems unlikely. This raises the question of what other possible means of stress communication may be functioning to explain the transmission of male-female stress influences. Regardless of the intriguing mysteries associated with these observations, it is relevant for cancer, nutritional, and other investigators to be aware of these potent proximity effects that are capable of altering various physiological parameters, including immunocompetence.

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SUMMARY

Experimentally or environmentally induced psychosocial stress produces increased levels of adrenal corticoids through well-known neuroendocrine pathways involving the cerebral cortex, the hypothalamus, the pituitary, and the adrenal cortex. A direct consequence of such stress-induced plasma corticoid elevation is injury to important elements of the immunological apparatus, which may leave the subject vulnerable to the action of latent oncogenic viruses, newly transformed cancer cells, or other incipient pathological processes that are normally held in check by an intact immunological surveillance apparatus.

In order to establish the potential pathological influence of alterations in adrenal corticoid levels, we have compared the biochemical and cellular effects resulting from experimental and environmental stress with those which occur following the imitation of "stress" through the direct administration of natural or synthetic corticoids. Both naturally occurring and chemically induced elevations of "stress" hormones produce similar physiological effects. In both cases, there is a lymphocytopenia, as well as a decrease in the weight of various immunological organs including the thymus, spleen, and peripheral lymph nodes.

The biochemical and physiological details of these processes have been examined in our laboratories using rodent models, with the expectation that the biological principles which apply to mice may be relevant in some respects to humans. Our attention is thus directed to an examination of the adverse influences that stress-induced elevated concentrations of circulating adrenal corticoids have upon thymus and thymus-dependent T cells, as well as their effects upon various neoplastic processes, including those involving viruses and transplantable malignant cells.

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Psychosocial stress can be quantitatively measured by both biochemical and cellular parameters, providing that truly quiescent baselines can be obtained in the control and experimental animals by the use of appropriate protective housing, modified handling procedures, and the elimination of common infectious contaminants.

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FUTURE STUDIES

The following areas of stress research are logical extensions of our current studies. It is traditional, as well as good research economy, to follow the most provocative leads produced by recent investigations.

Our earlier studies make it appropriate to carry out systematic studies on the role of the adrenal medulla in stress. These studies will be analogous in some respects to those that have been performed on the pituitary-adrenal-thymus pathways, involving primarily the adrenal cortex. Where feasible we shall use the methodologies that have been developed for adrenal cortex-associated stress. We hope to characterize the responses of the adrenal medulla in respect to the production of the highly active catecholamines as a consequence of quantitated stress. Such responses shall be compared and hopefully differentiated from stress that is primarily associated with activation of the adrenal cortex.

In terms of the developmental state of our studies, the time is now appropriate to apply some of the mouse findings to analogous studies using small primates. These studies will be carried out in collaboration with Dr. George Santisteban, using a colony of 54 squirrel monkeys. Efforts will be directed toward establishing quantitative values for the most critical parameters associated with stress and quiescence in primates in the same way that we have for mice.

An essential technique that is required for such primate studies is the establishment of non-invasive procedures for the quantitative measurement of various stress parameters. Examples would be establishing authentic cortisol levels under both quiescent and moderately stressful environments and circumstances.

In this connection, our recent studies with mice have laid the basis for measuring a specific stress-associated component in urine. Further development

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of this methodology would permit both daily and nocturnal stresses to be integrated in 12 and 24 hour urine collections, or other appropriate time intervals.

Figure 18 shows a fluorescent chromatograph peak (No. 4) that has been found in the urine of mice subjected to mild anxiety-inducing rotational stress. In the anticipation of applying stress studies to primates and humans, we have carried out preliminary studies to determine whether stress-associated metabolites could be detected in urine, and thus provide a non-invasive and integrative procedure for measuring stress. Using fluorescence analytical procedures developed originally for the plasma assays, we have shown the presence of substantial amounts of fluorescence-positive materials in urine. By employing column chromatographic separation methods, we have been able to demonstrate the presence of several fluorescence-positive components in mouse urine. Using procedures similar to those employed for the chromatography of plasma samples, we have separated four components from 24-hour collections of mouse urine, giving the results shown in Figure 18. The size of Peak 4 was in direct proportion to the plasma corticosterone level determined at the end of the urine collection period. Peak 4 is definitely not corticosterone, which is not present in mouse urine. This peak is probably a metabolite of corticosterone formed in the liver. We plan to further investigate the correlation of this peak with circadian levels of plasma corticosterone measured every 3 or 4 hours, to determine the reliability with which this peak might provide an integrated measure of the stress-related corticoid plasma levels during the 24-hour collection period.

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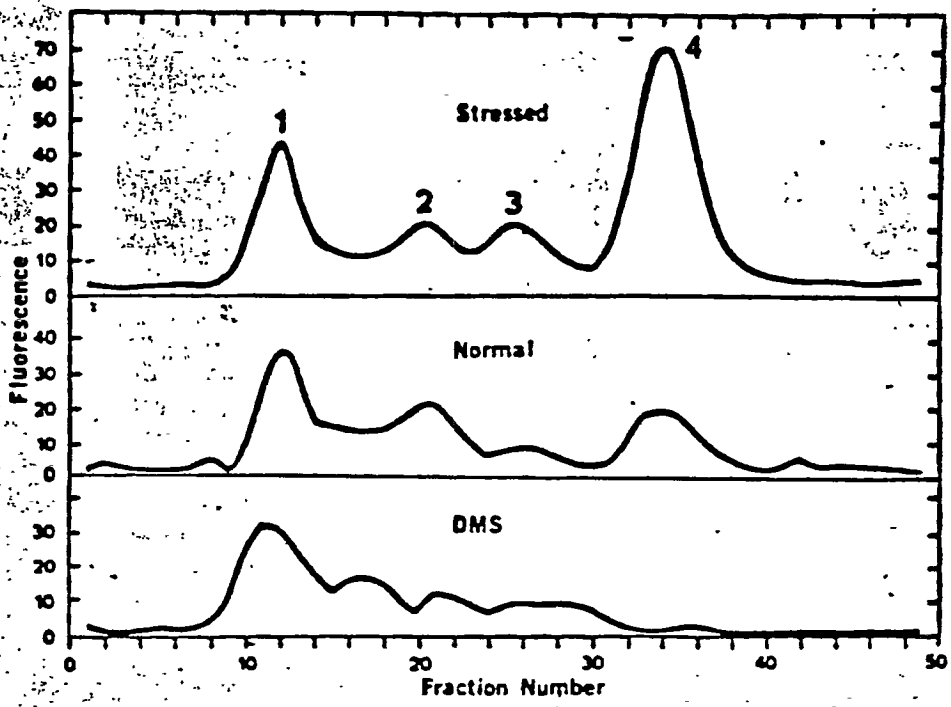

Figure 18

Fluorescent chromatographic peaks found in the urine of normal mice compared with similar animals experiencing mild anxiety-stress, and mice treated with dexamethasone (DMS) which blocks the endogenous production of corticosterone. Thus, this non-invasive technique may be applicable to the study of stress status in various experimental animals, possibly including man.

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Another non-invasive technique for measuring the effects of stress employing urine is the concentration-prioritized amino acid profile, which was developed in this laboratory. The utility of this technique is demonstrated by Figure 9, which shows the amino acid changes that occurred in mouse urine following mild rotation-induced stress. These changes may be related to the alterations observed in corticoid metabolites in the urine following stress. Thus, we are in the process of developing, refining, and correlating two separate non-invasive parameters for the measurement of stress-induced physiological changes, based upon measurements of substances found in the urine. These techniques, with appropriate modifications, can be applied to mice, rats, monkeys, and eventually, to man.

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FUTURE STUDIES

It is well known that multiple neuroendocrine responses occur in relation to emotional or anxiety stress. For example, in addition to plasma cortisol or corticosterone, increases also occur in plasma levels of epinephrine, norepinephrine, thyroxine, sex hormones, growth hormone, prolactin, insulin, and undoubtedly many others. In our investigations up to this point, for both scientific and practical considerations, we have performed stress studies with a special focus on the adrenal cortex system. Because of the encouraging clear-cut experimental findings obtained with the protective low-stress housing facilities and the application of quantitative techniques, it is now appropriate to enlarge our experimental scope, and to extend the present, intentionally limited, boundaries by measuring more of the vital parameters associated with the complex biochemistry of stress and its multifaceted physiological syndrome. We propose to do this by assaying other relevant hormones and biochemical substances that may be altered under conditions of controlled stress. Some of these components will be associated with the adrenal medulla.

Following are new areas of research that follow logically from the experimental base that has been established by our laboratory through the application of quantitative biochemical and cellular techniques employing mouse models in the study of physiological stress.

* Stress control. (Prophylaxis/therapy).
* Primate studies. (Squirrel Monkeys).
* Non-invasive sampling techniques. (Detection and quantitation of stress-induced metabolites in urine).
* Immunocompetence and stress. (Expansion of studies concerning the thymus, T cells, B cells, and macrophages).
* Pathological consequences of stress. (Further examination of the effects of stress upon the enhancement of various disease processes).
* Additional parameters. (Increase the number of hormones and physiological biochemicals to be monitored).
* The analysis and synthesis of data obtained from the various studies.
* Utilization of the 30 to 50 amino acids in plasma and urine for the characterization of important physiological changes that occur in the free and bound non-protein amino acids.

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Stress Control: Therapy or Prophylaxis.

These studies will be initiated using our established mouse models; as significant leads are obtained they will eventually be applied to primates maintained under low-stress and controlled stress environments.

Examples of stress prophylaxis and therapy experiments are as follows:

a) Use of specific enzyme inhibitors such as metyrapone (methopyrapone, metopirone), which inhibits corticosterone synthesis by blocking 11-B-hydoxylase. Metyrapone prevents a number of the usual manifestations of the stress syndrome, including adrenal hypertrophy and thymus involution.

b) Administration of various steroids, such as deoxycorticosterone, which may prevent some of the adverse effects of stress upon the immune system by successfully competing for cell receptor sites required by the stress-induced hormones. Dehydoepiandrosterone (DHEA) is another hormone-like compound whose physiological effects upon aging mice make it a logical candidate for stress-blocking studies.

c) Vitamin A has been reported to have blocking capabilities, which protect against some of the adverse physiological effects of either psychosocial stress or the direct administration of adrenal corticoids. Vitamin A and its retinoid analogs thus deserve a careful examination and re-evaluation under the controlled and quantitative experimental conditions that our laboratory can provide.

Primate Studies.

If support and resources are adequate, we plan to apply the techniques developed for the study of stress in rodents to non-human primates. More specifically, it is our intention to study the biochemical and cellular consequences of controlled anxiety stress in the Squirrel Monkey.

Such studies will be correlated with a parallel development of non-invasive techniques for the detection and quantitation of biochemical elements associated with psychosocial or anxiety stress. Examples of these techniques include the establishment of a special "metabolic" unit that communicates with the regular primate housing cage. The purpose of such an auxillary unit will be the collection of non-contaminated urine specimens under low-stress conditions in order to establish the quiescent base-line levels of various stress-associated hormones and other biochemicals. These quiescent levels may then be compared with the anticipated elevated levels associated with the quantitatively controlled induction of stress, and correlated with the levels of plasma cortisol.

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Stress and Immunocompetence.

Because of the important interrelationship between stress and immunological competence, it is also relevant to further increase our understanding of the details of this phenomenon. This may be accomplished by a more definitive identification of the specific sub-sets of lymphocytes that may be either adversely affected by the biochemical changes which occur during and following stress responses, or have a conspicuous resistance to the physiological changes associated with stress.

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FUTURE CONTINUATION STUDIES

To continue studies on establishing authentic concentrations of corticosterone in the plasma of normal and stressed animals.

The data shown in the attached Figures demonstrate the fallacy of the standard fluorescent assay for cortisol and corticosterone since the method incorporates the non-specific fluorogens. Unfortunately, these contribute significantly to the background values of the assay, and it thus does not measure true cortisol or corticosterone.

However, the data of Table 1 and the various related Figures demonstrate that it is possible to segregate and to measure the non-specific fluorescent peaks; and, of special significance, that these peaks represent a constant under a wide variety of experimental circumstances. Thus, this non-specific background can be subtracted from the assay by a single number; specifically, the equivalent of 22 ng/ml of corticosterone. These findings thus permit a significant correction to be made to corticosterone data, which in turn provides a more favorable circumstance for determining the true levels of circulating corticosterone in the plasma of animals under both quiescent and stressful conditions.

To continue studies on the correlation of unknown fluorescent peaks found in the urine of mice, with the stress-induced elevations of corticosterone in their plasma.

In order to study stress in an objective and effective way, a non-invasive assay has been suggested as the ideal solution by investigators. Our preliminary data demonstrates that there are fluorescence peaks of unknown composition in the urine of mice that are well-correlated with the degree of stress of the animals, and with the corresponding low or high levels of corticosterone in their plasma. This may provide the basis for a means of detecting stress without the necessity of bleeding the experimental animals. It also provides an integrated indication of the stress which occurs on a 24-hour diurnal basis. This is in contrast to the specific concentration of corticoids found in the plasma at the moment of any given bleeding.

If these findings are confirmed and can be applied to other species, including humans, it will provide an experimental means for a more versatile testing of both animals and patients for their stress status. Since the invasive techniques of venipuncture or other distressing procedures are stressful in themselves, the use of urine as an assay has obvious advantages. Thus, these findings will be pursued, and a determination made regarding their practical applications.

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To establish the chemical nature of the No. 4 fluorescence peak found in the urine of normal and stressed mice.

This specific fluorescence peak has been demonstrated to be correlated with the changing levels of corticosterone in the plasma of normal and stressed mice. Our chromatographic data establishes that this peak is not free corticosterone, and thus must be a conjugate or metabolic product of corticosterone. It is thus desirable to try to identify this material. Appropriate experiments will be undertaken and efforts will be made to establish its nature by substituting known possible candidates, and by submitting the peak fraction to appropriate chemical procedures in an effort to return it to its original free corticosterone state. If identification is not provided by these simple straightforward procedures, additional efforts will be made to establish its identity by gas chromatography, mass spectrophotometry, or other appropriate techniques.

To examine the urine of various species for the presence of fluorescence peaks that may correlate with anxiety or psychosocial stress.

Although the primary experiments in this proposal will employ mice, where the findings suggest possible applications to human beings, this will be approached experimentally by examining the urine of other species, such as rats, dogs, and primates. These experiments will be designed to determine whether there are similar fluorescent correlates in the urine of the various species, and whether their concentration is altered as a function of anxiety or induced psychosocial stress. In the event that encouraging findings are obtained with several non-human species it would then be appropriate to consider its application to patients or other individuals undergoing stress in comparison with the more normal and quiescent subjects.

To employ the broad spectrum of free and bound amino acids that are present in the plasma and urine, as a multi-parameter index of metabolic alterations that occur as a consequence of exposure to anxiety stress.

In order to relate stress and associated metabolic changes, it is necessary to establish the relative concentrations of the non-protein (free and bound) amino acids in the plasma, urine, and relevant tissues and organs of mice, rats, and possibly primates, to serve as reference values for biological stress model studies; and to organize such quantitative concentration values into prioritized Amino Acid Profiles in order to visually demonstrate any significant difference between samples. By the determination of such Amino Acid profiles, it is feasible to study the changes which take place in the metabolism of these amino acids under the influence of stress-elevated plasma levels of corticosteroid hormones. Such measurements will include both high levels of naturally occurring corticosterone concentrations resulting from controlled stress, as well as the stress-simulated effects of injected natural and synthetic corticosteroids.

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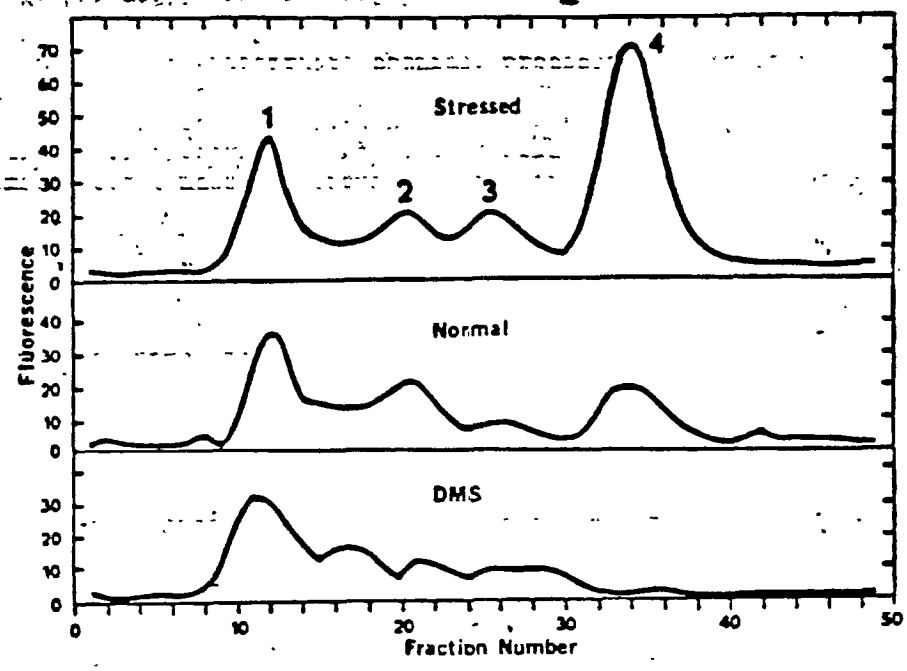

Fluorescent chromatographic peaks found in the urine of normal mice compared with similar animals experiencing mild anxiety-stress, and mice treated with dexamethasone (DMS) which blocks the endogenous production of corticosterone. Thus, this non-invasive technique may be applicable to the study of stress status in various experimental animals, possibly including man.

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TABLE I

FLUORESCENCE ANALYSIS OF MOUSE URINE COMPARED TO PLASMA

Experimental Conditions of Mice	Plasma Corticosterone; End of 24 Hrs., A.M., W-Assay[1]	Urine Assay[2]		
		W-Assay[1]	C-Assay[3] Peak 4	Total Peak No. 4 per Mouse per 24 hrs.
Rotation-Stressed	351	271	165	330
Normal	38	214	51	102
DMS-Treated	30	171	4	8

1. Whole plasma or whole urine assay.

2. 24-hour collections of urines acidified and reconstituted to 2 ml per mouse per 24-hours.

3. Fluorescence assay after chromatographic separation.

All values expressed in ng/ml as corticosterone; urine components could be more or less than the given values depending on their fluorescence relative to corticosterone. Mice are BDF or C3H females, 8-12 weeks old.

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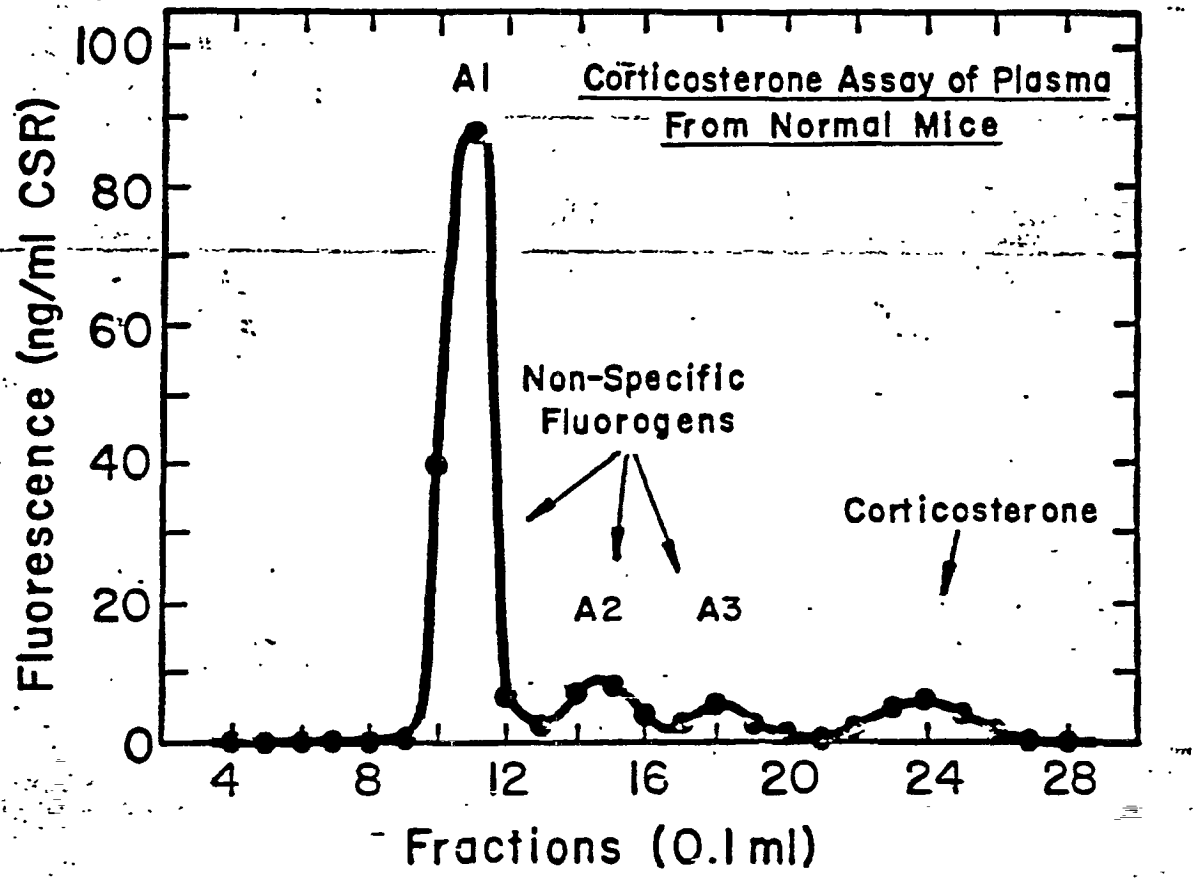

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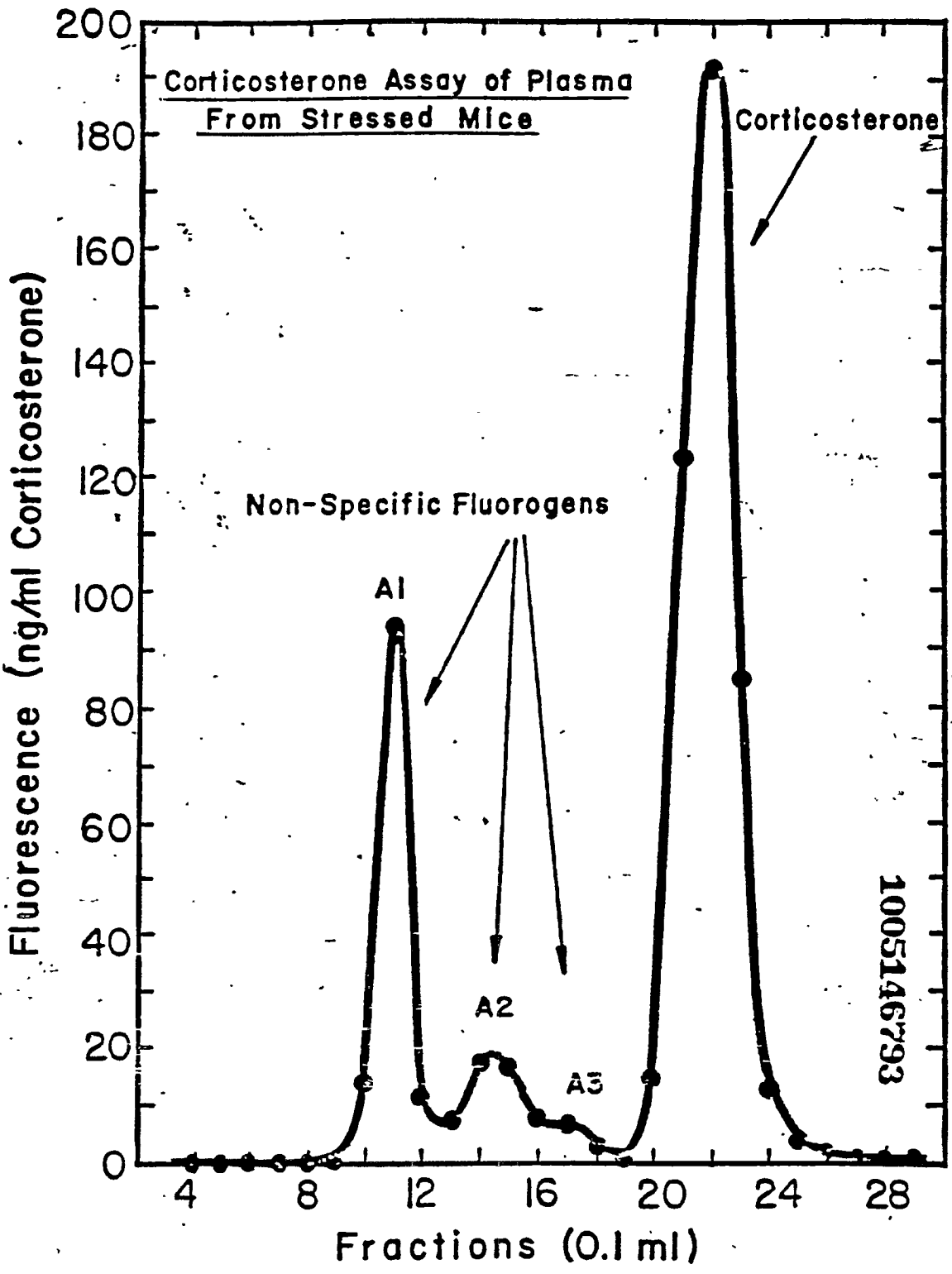

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R: REDACTED MATERIAL

BIOGRAPHICAL INFORMATION

(Give the following information for all professional personnel listed on page 1, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	REDACTED (Yr.)
Riley, Vernon T.	Chairman, Department of Microbiology	
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	REDACTED
Idaho Falls, Idaho, U.S.A.	U.S. Citizen	☒ Male ☐ Female

EDUCATION *(Begin with baccalaureate training and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Spokane College, Spokane, Washington	A.B.	1935	Biology
George Washington University			Biology
University of Paris (Sorbonne), France	D.Sc.	1966	Microbiology

HONORS

Doctoral thesis, Tres Honorable, Sorbonne
Fellow, New York Academy of Sciences; Chairman, International Pigment Cell Conferences (1962-1975); National Member, Society for Experimental Biology and Medicine.

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Basic physiological processes involving cell biology, enzymology, and virology	Principal Investigator

RESEARCH SUPPORT *(See instructions)*

NCI Grant entitled, INFLUENCE OF ANIMAL HANDLING TECHNIQUES UPON THEIR PHYSIOLOGICAL STATE; R for the time period from 3/01/78 to 2/28/79.

USAFSAM Contract No. F 33615-77-C-0617, entitled, RF RADIATION EFFECTS ON CELLS; R for the time period from 5/27/77 to 5/26/78.

USAFSAM Contract No. F 33615-77-C-0623, entitled, BIOLOGICAL CONSEQUENCES OF PULSED VERSUS CONTINUOUS WAVE RADIOFREQUENCY R for the time period from 7/27/77-7/26/78.

ACS Grant entitled, BREAST CARCINOMA: ROLE OF PHYSIOLOGICAL STRESS; R 1/1/77-12/31/78

RESEARCH AND/OR PROFESSIONAL EXPERIENCE *(Starting with present position, list training and experience relevant to area of project, including teaching and clinical responsibilities. List all or most representative publications.*

Chairman, Department of Microbiology, Pacific Northwest Research Foundation, Seattle, Washington, September 1967 to present.
Member, Fred Hutchinson Cancer Research Center, Seattle, Washington, June 1973 to present.
Full Professor, Rehabilitation Medicine, University of Washington School of Medicine (adjunct), 1976 to present.
Full Professor, Experimental Animal Medicine, University of Washington School of Medicine (affiliate), June, 1971 to 1973.
Head of Section, and Head of Laboratory, Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, Rye, New York, 1954-1967; Assistant 1954-60; Associate, 1960-67.
Biologist, National Cancer Institute, National Institutes of Health, U.S.P.H.S., Bethesda, Maryland, 1943-1953.
Senior Research Assistant in Chemistry (Oceanographic Laboratory), University of Washington (Fermentation Research), 1937-40.
Assistant Bacteriologist, Hollister-Stier Laboratories, Spokane, Washington, 1935-1936.
The above experiences included 5 years training in quantitative virology with W. Ray Bryan (NCI), and 5 years training in enzymology and cellular metabolism with Drs. Dean Burk, Mark Woods, Jesse Greenstein, and Otto Warburg.
The principal investigator was a visiting scientist with Prof. Charles Oberling, Villejuif, France, 1959; and with Prof. Raymond Latarjet and Dr. Robert Cramer, Laboratoire Pasteur, de l'Institut de Radium, Paris, 1960.
The principal investigator received his doctorate in microbiology under the Nobel laureate, Andre Lwoff, Pasteur Institute, and University of Paris.

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ADDITIONAL HONORS:

- Harvey Society; Series Editor of Pigment Cell Monographs, S. Karger, Basel;
  Listed in the following publications:
  WORLD WHO'S WHO IN SCIENCE
  AMERICAN MEN OF SCIENCE
  AMERICAN MEN AND WOMEN OF SCIENCE

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   AAAS Research Conference on Cancer, 40-53 (1944)

2. Studies on the Influence of Age on the Latent Period Response of Chickens to the Agent of Chicken Tumor I.
   Vernon T. Riley, Dorothy Calnan and W. Ray Bryan
   J. Nat. Cancer Inst., 7:93-98 (1947)

3. Absence of Effect of Urethane on the Latent Period of Tumors Induced with Subcutaneous Injections of the Agent of Chicken Tumor I.
   W. Ray Bryan, Vernon T. Riley and Dorothy Calnan
   J. Nat. Cancer Inst., 7:203-206 (1947)

4. Studies on the Agent of Chicken Tumor I. I. The Relative Potencies of Serial Extracts and their Comparative Values as Source Materials for Purification Studies.
   W. Ray Bryan, Vernon T. Riley, D. George Deihl, and Virginia Voorhees
   J. Nat. Cancer Inst., 7:447-453 (1947)

4a. Studies on Purification of the Agent of Chicken Tumor I.
   W. Ray Bryan and Vernon T. Riley
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5. Application of Chromatography to Segregation Studies of the Agent of Chicken Tumor I. (Rous Sarcoma Virus)
   Vernon T. Riley
   Science, 107:573-575 (1948)

6. Application of Chromatography to the Separation of Subcellular Enzymatically Active Granules.
   Vernon T. Riley, M. L. Hesselbach, Silvio Fiala, M. W. Woods and Dean Burk
   Science, 109:361-364 (1949)

6a. Application of Chromatography to the Segregation of Subcellular Particulates.
   Vernon T. Riley, M. L. Hesselbach, M. W. Woods and Dean Burk
   Cancer Research, 9:614 (1949)

7. A Short Column Procedure for Separating Cytoplasmic Components from Normal and Tumor Tissue.
   Vernon Riley and M. W. Woods
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8. Chromatographic Separation of Melanin Granules.
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10. Chromatographic Studies on the Separation of the Virus from Chicken Tumor I. I. Effect of Salt Concentration on Adsorption, Elution, and Purification.
Vernon Riley
J. Nat. Cancer Inst., 11:199-214 (1950)

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Vernon Riley
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VOGUE

STRESS AND CANCER

BY DR. ELIZABETH M. WHELAN

Are you under a great deal of stress at work? Have you any idea how much you can safely take? What's your blood pressure reading? When was the last time you had it checked? Far too few of us are aware of the killing toll stress takes.

Stress is widely recognized as a causative factor in a number of diseases, notably ulcers and hypertension. But now there is a new and dramatic question being asked: Is stress an important link in cancer development? Can the way you handle stress (for instance, stifling your feelings) affect your chance of developing this disease? Very possibly, according to evidence presented at the Third International Symposium on Detection and Prevention of Cancer held recently in New York City.

Right now, medical scientists are looking closely at three related questions. First, what effect may traumatic events such as the loss of a loved one or a huge financial loss have on susceptibility to cancer? Second, is there a "cancer-prone" personality—a type of person who deals with life stresses in a manner which predisposes him or her to cancer? Third, how may the biological changes which occur when we are under stress affect the ability of our body's defense system to protect us from cancer-causing agents?

● STRESS AND SUSCEPTIBILITY TO DISEASE

No day goes by without all kinds of stress—driving to work, playing tennis, catching a train, giving a dinner party. Even waking up in the morning is a form of stress which jars our fully relaxed bodies. But the stress of interest to a small but growing group of cancer researchers is beyond the everyday variety. It is the type that leads to deep depression, constant stomach-churning anxiety, prolonged frustration.

The fact that unusually stressful events or lifestyles can predispose us toward disease is well known. For instance, many studies have shown that air traffic controllers, who are under constant stress, have a much higher rate of high blood pressure, stomach ulcers, diabetes and other diseases than do people in less demanding airline positions. Clinicians are constantly impressed by the influence of the psyche on susceptibility to physical disease.

Theories linking stress and cancer have been around for a long time. In the 1800s the renowned physician Sir James Paget wrote: "The cases are frequent in which deep anxiety, deferred hope and disappointment are quickly followed by the growth or increase of cancer . . ."

A classic study done during the 1960s lent some modern supporting data to that 19th-century observation. William A. Greene, a psychiatrist at the University of Rochester, studied the life history of three sets of twins. One twin out of each set developed leukemia. He found that these three had experienced a psychological upheaval right before the onset of the disease. The three others had undergone no similar stress. Dr. Green concluded that psychological trauma might well be a precipitating factor in cancer.

Dr. H. J. F. Baltrusch, a researcher from the International Psychosomatic and Cross-Cultural Leukemia Project in West Germany, reported to the cancer symposium on a study of more than 8,000 patients with different types of cancerous lesions. "In the majority of patients," he said, "clinical manifestations of malignancy occurred during a period of severe and intensive life stress frequently involving loss, separation and other bereavements."

● IS THERE A CANCER-PRONE PERSONALITY?

Severe life stress may be important in bringing about or promoting cancer, and so may be the way we handle that stress. From 1948 to 1964, Dr. Caroline B. Thomas collected physical and psychological profiles of 1,337 medical students at Johns Hopkins. She kept track of them by means of yearly questionnaires and recorded the cause of death if it occurred. Dr. Thomas found that cancers tended to develop in people who were generally quiet, nonaggressive and emotionally contained.

Dr. René C. Mastrovito, a psychiatrist from the Department of Neurology at Memorial Sloan-Kettering Cancer Center in New York, presented at the cancer symposium additional evidence of a cancer-prone personality. In a detailed psychological study of women admitted for gynecological checkups, he found that women with cancer were highly controlled and conforming, and less adventurous, assertive, competitive and spontaneous than those whose cancer tests were negative. Dr. Mastrovito's observations were bolstered by those of Dr. S. Greer from King's College Hospital in London. He reported a significant link between breast cancer and abnormality in the release of emotions, particularly extreme suppression of anger and a bottling up of feelings.

But exactly how could the stress of emotional trauma—or repression of anger and other emotions—affect the probability of cancer development? What type of biology could be involved?

● MICE, MEN AND CANCER

Some recent animal experiments offer possible answers. Dr. Vernon Riley and his colleagues, Drs. Darrel Spackman, George Santisteban and Heather McGlanahan of the Pacific Northwest Research Foundation in Seattle, have demonstrated that stress brings about physiological changes which affect susceptibility to cancer. In one experiment, they selected a strain of mice which is highly cancer-prone. Under most conditions, 80 to 100 per cent of these animals develop tumors within 8 to 18 months after birth. But when the researchers put them behind a protective barrier, keeping them free of the normal laboratory noise and other commotion which might generate anxiety and stress, only 7 per cent developed cancer after 14 months.

In another experiment with laboratory animals, Dr. Riley showed that rotating them gently on a turntable was enough to lead to higher cancer rates.

The stress evidently brin about significant hormon changes which interfere wi the animals' immunologic defense system against ca cer. Are there any implic tions for humans in this r search? Dr. Riley and othe think there are.

If we are in good heal our "cancer surveillanc system is operating effecti ly. Many researchers n feel that we encounter ca cer-causing agents every d but are not affected by thi because our immune syst rejects them before they c begin the cancer growth pr cess.

If we follow the sa stress response pattern animals (and so far we ha no reason to believe that don't), it appears that hea or prolonged stress can i terfere with that surveillar system. Our adrenal and tuitary glands produ ACTH, cortisone and oth hormones which stimul certain body reactions. So of these stress-induc chemical changes proba cause no ill effects—the bo still goes on fighting off ca cinogens. But if the syst gets overloaded there may serious trouble. Indeed, e cessive amounts of those pe fectly natural physiologi changes which accompa stress may damage our i munological apparatus to t extent that we are left v nerable. It may be for onl very short period of time, b even that period may crucial.

● STRESS, CANCER AND YOU

If stress does predispose to cancer, what are we goi to do about it? Researchers are now taking a close lo at those alterations in t cancer-resistance syst that may be induced stress, with the hope of co ing up with some type blocking agent which wo keep the body on an e hormonal keel despite t aggravating external ever Ideally, such blocking ag

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"Severe life stress may be important in bringi about a cancer, and so may be the way we hand that stress."

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STRESS AND CANCER

CONTINUED FROM PAGE 105

would keep our defense system in continuous working order so that cancer-causing agents are thwarted—in a continuous, uninterrupted manner.

Another approach is psychological screening techniques to identify high-risk patients *before* a cancer actually develops. Drs. Claus and Marjorie Bahnson, a husband-wife psychology team at the Eastern Pennsylvania Psychiatric Institute, have developed a questionnaire which covers topics such as loss and reactions to loss of close relatives, stress and recent life changes, personality characteristics and means of handling stress. They feel this research-diagnostic tool can help identify potential cancer victims early, in time for prevention or effective treatment.

In the meantime, Dr. Riley and others feel we can act on the knowledge collected to date. Perhaps there is indeed something therapeutic—and advantageous for the maintenance of our disease-surveillance mechanism—about ventilating feelings, instead of keeping them all inside.

We could also learn something from the Mormons and Seventh Day Adventists—two groups with unusually low incidences of cancer. True, most neither smoke nor drink and may eat differently from the rest of us. But cancers that have nothing to do with smoking, drinking or eating are also low. Does it have something to do with their attitude toward life—their more serene outlook?

"Could be," says Dr. Riley. "They have closer family relationships, too—and generally less external stress. All we know is that if you want to minimize your chances of cancer, give up cigarettes, eat less fatty food, keep thin—and live the most serene lifestyle circumstances permit."

He adds, "I know that's a big order. But we are learning more and more about the possible link between stress and cancer. It's getting to be a very exciting field."

Dr. Riley is obviously enthusiastic about his research—but he is also quite relaxed.

EDITOR'S NOTE: Dr. Elizabeth M. Whelan is the co-author, with Dr. Fredrick J. Stare of *Panic in the Pantry* (Atheneum) and author of *A Baby? Maybe: A Guide to Making the Most Fateful Decision of Your Life* (Bobbs-Merrill). She is currently working on a book on cancer and the environment.

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BIOGRAPHICAL SKETCH

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Nationality:

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Education:

Weber College, Ogden, Utah	1946-1948	Diploma
University of Utah, Salt Lake City	1948-1950	B.A. (Chemistry)
University of Utah, Salt Lake City	1950-1952	M.A. (Biochemistry)
University of Utah, Salt Lake City	1952-1954	Ph.D. (Biochemistry)

Military Service:

United States Army Air Force, 1943-1946 (United States, South Pacific, Japan).

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Major Research Interest:

The isolation, determination and characterization of amino acids, peptides and proteins, particularly as related to malignant processes; and methodology for the automatic analysis of amino acids.

Research and Professional Experience:

Present Position

Senior Research Biochemist, Department of Microbiology, Pacific Northwest Research Foundation, Seattle, Washington, 1968 to present. Research Scientist, Fred Hutchinson Cancer Research Center, Seattle, Washington, 1975 to present. Professor, Department of Rehabilitation Medicine, University of Washington School of Medicine (adjunct).

Previous Experience

Research Assistant Professor of Obstetrics-Gynecology and of Biochemistry, University of Washington, Seattle, Washington, 1963-1968.

Senior Biochemist, Spinco Division, Beckman Instruments, Inc., Palo Alto, California, 1959-1962.

Research Associate, The Rockefeller Institute, New York, New York, in the Department of Drs. Stanford Moore and William H. Stein, 1954-1959. Drs. Moore and Stein were awarded the Nobel Prize in chemistry in 1972.

Postgraduate Fellowship, United States Public Health Service, under Dr. Emil L. Smith at the University of Utah, 1951-1954; received doctorate in biochemistry.

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SECTION II - PRIVILEGED COMMUNICATION

BIOGRAPHICAL SKETCHES

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME	TITLE	BIRTHDATE (Mo., Day, Yr.)
Santisteban, George Anthony	Senior Biologist	REDACTED

PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX
Cananea, Sonora, Mexico	U.S. Citizen	☒ Male ☐ Female

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Montana State University, Missoula, Mon.	B.A. (honors)	1945	Zoology
University of Utah, Salt Lake City, Utah	M.A.	1949	Zoology
University of Utah, Salt Lake City, Utah	Ph.D.	1951	Anatomy
University of Utah, Salt Lake City, Utah	Postdoctoral	1951-53	

HONORS

American Associatipn for Cancer Research, American Association of Anatomists, Phi Sigma Biological Honorary Society, and New York Academy of Sciences

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Stress, adrenocortical-thymus function and malignancy.	Collaborator

RESEARCH SUPPORT (See instructions)

NIH Grant # 5 RO1 NS 09758, SOCIAL STIMULATION, RNA, AND CARDIOVASCULAR DISEASE. REDACTED 50% of time involved.

This grant expires August 31st, 1976). Renewed 9/1/76.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

Professor of Biology, Seattle University, Seattle, Washington 1974 to present.
Affiliate, Fred Hutchinson Cancer Research Center, Seattle, Washington 1973 to present.
Senior Biologist, Pacific Northwest Research Foundation, Seattle, Washington, 1968 to present.
Associate Professor of Biology, Seattle University, Seattle, Washington, 1968 to 1974.
Acting head, Department of Biology, Seattle University, Seattle, Washington, 1966 to 1968.
Associate Professor of Biology, Seattle University, Seattle, Washington, 1964 to 1968.
Special Fellowship, U.S.P.H.S., to continue training and do research at the University of Gothenburg (Goteborg), Sweden, under sponsorship of Professor Holger Hyden, 1966 to 1967.
Research Associate at University of Gothenburg, Sweden, to obtain special training in the microanalytical methods for studying intracellular RNA, under sponsorship of Professor Holger Hyden. (On leave from University of Southern California School of Medicine) 1963 to 1964.
Assistant Professor of Physiology (Neurophysiology). University of Southern California School of Medicine, 1959 to 1963.
Assistant Professor of Anatomy (Neuroanatomy, Microscopic Anatomy), University of Southern California, 1954 to 1959.
Assistant Professor (Gross Anatomy). Medical College of Virginia, Richmond, Virginia, 1953 to 1954.

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Riley, Vernon T.

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Postdoctoral Training (Research Instructor) under Dr. Thomas F. Dougherty; Cancer biology, radiobiology, biology of lymphatic tissue and studies of the lymphocytolytic effects of the adrenocortical steriod hormones, 1951-1953.

Lecturer in Anatomy (Elementary Anatomy, gross anatomy, neuroanatomy, medical histology), University of Utah, School of Medicine, 1949-1951.

Teaching Assistant in Anatomy (Elementary anatomy, gross anatomy, neuroanatomy, medical histology), University of Utah, School of Medicine, 1947-1949.

Graduate Assistant in Zoology (Comparative vertebrate zoology and mammalian anatomy), Department of Zoology, University of Utah, 1946-1947.

Assistant in Zoology (General Zoology and comparative vertebrate anatomy), University of Montana, Department of Zoology, 1945-1946.

Student Assistant in Zoology, Department of Zoology, University of Montana, 1944 to 1946.

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Riley, Vernon T.

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Santisteban, G.A.: Influence of age, sex, and post-operative time interval on the growth of lymphatic tissue following adrenalectomy. Abst. Anat. Rec. 123:2 p. 357 (1956).

Santisteban, G.A., and Swinyard, C.A.: The effect of ethyl alcohol on adrenal cortical activity in mice. Endocrinology 59:4, pp. 391-397 (1956).

Santisteban, G.A.: The influence of the adrenal cortex on the regrowth of lymphatic tissue following the acute involution induced by ethyl alcohol. Abst. Anat. Rec. 127:2 (1957).

Santisteban, G.A.: Studies on the relationship of the acute involution of lymphatic tissue to the severity of stress stimuli. Abst. Anat. Rec. 130:2, p. 368 (1958).

Santisteban, G.A.: Adrenocortical influences upon the reconstitution of the thymus and lymph nodes following acute involution. Endocrinology 64:5, pp. 638-647 (1959).

Santisteban, G.A.: The correlation of the growth of lymphatic tissue with body growth and the growth of the adrenal gland and genital tissue in CBA mice. Abst. Anat. Rec. 136:2, p. 331 (1959).

Santisteban, G.A.: The alterations of the lymphocytolytic activity of cortisol by blood. Abst. Anat. Rec. 134:2, p. 398 (1959).

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R: REDACTED MATERIAL

Riley, Vernon T.

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Santisteban, G.A.: Comparison of the influences of various forms of stress stimuli upon the adrenocortico-thymico-lymphatic system in CBA mice. Abst. Anat. Rec. 134:2, p. 424 (1959).

Santisteban, G.A.: The influences of sex upon the resistance and the adreno-cortico-thymico response to acute alcohol intoxication in CBA mice. Abst. Anat. Rec. 134:2, p. 424 (1959).

Santisteban, G.A., and Dougherty, T.F.: Growth hormone treatment following whole-body X-irradiation in mice. Abst. Anat. Rec. 134:2, p. 423 (1959).

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Riley, Vernon T.

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